Nectin ectodomain structures reveal a canonical adhesive interface

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Nectins are immunoglobulin superfamily glycoproteins that mediate intercellular adhesion in many vertebrate tissues. Homophilic and heterophilic interactions between nectin family members help mediate tissue patterning. We determined the homophilic binding affinities and heterophilic specificities of all four nectins and the related protein nectin-like 5 (Necl-5) from human and mouse, revealing a range of homophilic interaction strengths and a defined heterophilic specificity pattern. To understand the molecular basis of their adhesion and specificity, we determined the crystal structures of natively glycosylated full ectodomains or adhesive fragments of all four nectins and Necl-5. All of the crystal structures revealed dimeric nectins bound through a stereotyped interface that was previously proposed to represent a cis dimer. However, conservation of this interface and the results of targeted cross-linking experiments showed that this dimer probably represents the adhesive trans interaction. The structure of the dimer provides a simple molecular explanation for the adhesive binding specificity of nectins.

Nectins comprise a small family of immunoglobulin superfamily cell adhesion proteins that contains four members, nectin-1 through nectin-4 (also known as poliovirus receptor-related proteins 1–4), that are conserved in vertebrate species1–4. Nectins are characterized by an ectodomain containing three tandem immunoglobulin-like regions arranged as an N-terminal variable-like domain (D1) followed by two constant-like domains (D2 and D3), a single transmembrane region and a cytoplasmic domain that binds to the actin cytoskeleton through the adaptor protein afadin5. Alternative splicing gives rise to several splice forms of nectin-1, nectin-2 and nectin-3, which differ from each other in their transmembrane and cytoplasmic regions6. A highly related family of proteins, referred to as nectin-like proteins 1–5 (Necl-1–Necl-5), shares a similar domain organization with the nectins but does not bind to afadin. Of the nectin-like proteins, Necl-1 through Necl-4 are highly similar to each other and probably represent a functionally distinct subfamily, also referred to as synaptic cell adhesion molecules (synCAMs)6. Necl-5 (also known as poliovirus receptor), although also unable to bind afadin, is more closely related to the nectins at the sequence level6,7. The present study focuses on the nectin subfamily and the highly related protein Necl-5.

Nectins mediate calcium-independent cell-cell adhesion in many vertebrate tissues, including epithelia, endothelia and neural tissue, during development and in adulthood3,8,9. Individual nectin subtypes are expressed in distinct but overlapping patterns such that a given cell may express one nectin or a combination of several nectins10. At the subcellular level, nectins are restricted to the subapical regions of lateral membranes in polarized epithelial cells, where their localization seems to overlap with that of zonula adherens components, including cadherins11–13. Nectin-1 through nectin-4 all mediate homophilic cell adhesion (between cells expressing identical nectin subtypes) and have been shown to drive cell aggregation when exogenously expressed in cells that are normally nonadherent11,12,14,15 and localize symmetrically at homotypic cell-cell contacts16–18. Additionally, nectins engage in heterophilic adhesion in specific pairwise combinations: heterophilic adhesion has been observed between nectin-1 and nectin-3, nectin-1 and nectin-4 and nectin-2 and nectin-3 in mixed cell aggregation assays, co-culture experiments10,12,19,20 and binding assays using purified proteins7,17,21,22. Notably, these heterophilic interactions seem to be markedly stronger than the respective homophilic interactions in these assays, resulting in larger cell aggregates20, higher resistance to separation force23 or higher amounts of bound protein17,21. Necl-5, unlike nectin-1–nectin-4, does not mediate the homophilic aggregation of transfected cells7,14 but does weakly bind heterophilically to nectin-3 (refs. 7,21,24).

Heterophilic binding between cognate nectin pairs, which is stronger than the homophilic binding of either subtype, seems to underlie a unique role for nectins in mediating heterotypic cell-cell associations10. Mice genetically null for individual nectins are viable and develop to adulthood, probably because of redundancy between nectin subtypes in most tissues10,25–27 but have developmental phenotypes that reveal nonredundant roles for the heterophilic interactions of nectins. Nectin-1 (Pvrl11−/−) and nectin-3 (Pvrl3−/−) knockout mice

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have microphthalmia resulting from phenotypically identical defects in the formation of the ciliary body of the eye, where heterophilic binding between nectin-1 and nectin-3 is required to link two distinct epithelial layers. Nectin-2 (Pvrl2Δ3/Δ3) and nectin-3 (Pvrl3Δ3/Δ3) knockout mice have male-specific infertility caused by defects in sperm development, for which heterophilic adhesion between nectin-2 expressed in the seminiferous epithelium and nectin-3 expressed in developing spermatids seems to be crucial. In human disease, mutations in the genes encoding nectin-1 and nectin-4 are associated with cleft lip and/or palate ectodermal dysplasia and ectodermal dysplasia–syndactyly syndrome, respectively. These diseases share several common symptoms, suggesting an important role for heterophilic interactions between nectin-1 and nectin-4 in the ectoderm.

Homophilic and heterophilic binding between nectins measured in cell aggregation assays and binding assays using purified proteins requires the membrane-distal immunoglobulin variable-like domains in all pairwise combinations. The crystal structures of the ectodomains of nectin-1, nectin-2, nectin-3, nectin-4 and Necl-5 in all pairwise combinations revealed the crystallographic dimer represented a cis dimer (between nectins on the same cell) rather than a trans dimer (between nectins on apposed cells), in part because the orientation of the interacting protomers was compatible with either a cis or a trans dimer and in part because mutation of the interface, in addition to abolishing adhesion, also diminished the proportion of nectin that could be chemically cross-linked as a dimer on the surfaces of dissociated transfected cells. Such cross-linking assays have been cited as evidence for the cis dimerization of nectin, as cells are dissociated and no trans bonds should form. On the basis of these data, the authors proposed a model for nectin adhesion in which nectins form cis dimers on the cell surface through the FGCC′C″ interface that are essential for subsequent trans binding through an interface that was not present in the crystals. Because the authors identified no candidate trans binding interface, the structural basis for nectin adhesion, and, consequently, for nectin adhesive specificity, has remained unresolved.

We undertook a biophysical and structural analysis of the nectin family (nectin-1–nectin-4 and Necl-5) to determine the molecular basis of nectin adhesion and specificity. We tested the binding between purified nectin ectodomains in all pairwise combinations and determined the equilibrium constants for the nectin homodimers. The crystal structures of ectodomains of nectin-1, nectin-2, nectin-3, nectin-4 and Necl-5 presented here identify a single conserved interface that we show, contrary to its previously suggested role as a cis dimer, to be the adhesive trans interface of nectins. Identification of the nectin adhesive interface provides a framework for understanding the structural basis of the specificity of nectin adhesive binding, which seems to be mediated in large part by electrostatic compatibility between cognate pairs.

RESULTS

Nectin ectodomains form homophilic and heterophilic dimers

We purified full-length, natively glycosylated ectodomains (D1–D3) of human and mouse nectin-1, nectin-2, nectin-3 and nectin-4 and human Necl-5 from conditioned media of transfected HEK 293F cells. We assessed the homophilic binding properties of the proteins in solution using equilibrium analytical ultracentrifugation (AUC). Human nectin-1–nectin-4 showed monomer-dimer equilibrium in solution, with apparent molecular weights in the AUC that were higher than the molecular weights of the monomers, as determined by mass spectrometry (Table 1). Fitting of the data to a 1:1 binding model yielded $K_d$ values for homodimerization that were in the micromolar range but that varied widely between the subtypes. The nectins fell into two groups: nectin-1 and nectin-2 formed relatively stronger homophilic dimers ($K_d$ values of 17.5 µM and 0.4 µM, respectively), whereas the dimerization of nectin-3 and nectin-4 was very weak ($K_d$ values in the hundred micromolar range). These relative affinities were conserved in mouse nectin-1–nectin-4 (which have 64–97% sequence identity to the human orthologs in the D1 domains), with tighter homophilic binding for nectin-1 and nectin-2 than for nectin-3 and nectin-4 (Table 1). Necl-5 did not dimerize detectably in solution, which is consistent with previous reports that Necl-5 cannot mediate homophilic cell-cell adhesion.

We used surface plasmon resonance (SPR) to test for heterophilic binding between the ectodomains (D1–D3) of human nectin-1–nectin-4 and Necl-5 in all pairwise combinations (Fig. 1). We detected specific heterophilic binding between nectin-1 and nectin-3 (Fig. 1a,c), nectin-1 and nectin-4 (Fig. 1a,d) and nectin-2 and nectin-3 (Fig. 1b,c). In addition, we detected a very low amount of binding between Necl-5 and either nectin-3 or nectin-1 (Fig. 1c,e). Other combinations of nectin subtypes yielded no detectable binding responses. The heterophilic interactions detected in our analyses are in agreement

| Table 1 Analytical ultracentrifugation analysis of nectin homodimerization |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Protein   | Monomer MW from mass spectrometry (kDa) | Apparent MW in AUC (kDa) | Oligomeric state | $K_d$ dimerization ($\mu$M) |
| Human     |                           |                           |               |                           |
| Nectin-1  | 43.3$^a$                  | 67.5 ± 0.79$^c$           | Dimer         | 17.5 ± 2.2$^c$            |
| Nectin-2  | 39.4                      | 75.8 ± 0.10$^c$           | Dimer         | 0.392 ± 0.029             |
| Nectin-3  | 47.5                      | 52.6 ± 0.077$^c$          | Weak dimer    | 228 ± 9.5                 |
| Nectin-4  | 35.6                      | 43.1 ± 1.2                | Weak dimer    | 153 ± 28                  |
| Necl-5    | 46.8                      | 45.7 ± 1.6                | Monomer       | ND                        |
| Nectin-1  | 43.6$^a$                  | 43.8 ± 0.099              | Weak dimer    | 820 ± 101                 |
| Mouse     |                           |                           |               |                           |
| Nectin-1  | 44.2                      | 65.3 ± 0.87               | Dimer         | 22.6 ± 1.3                |
| Nectin-2  | 40.2                      | 75.7 ± 0.11               | Dimer         | 1.09 ± 0.10               |
| Nectin-3  | 57.6                      | 66.4 ± 1.2                | Weak dimer    | 122 ± 16                  |
| Nectin-4  | 36.1                      | 42.1 ± 2.1                | Weak dimer    | 278 ± 137                 |
| Necl-2    | 28.5                      | 49.1 ± 0.17               | Monomer       | 7.62 ± 0.016              |
| Necl-2    | 26.4                      | 24.4 ± 0.50               | Monomer       | ND                        |
| Heterocomplexes (human, D1–D3) |                       |                           |               |                           |
| Nectin-1 + nectin-3 | 43.3 + 47.5$^c$         | 86.7 ± 0.08              | Dimer         | ND                        |
| Nectin-1 + nectin-4 | 43.3 + 35.6$^c$           | 77.4 ± 1.0               | Dimer         | ND                        |

$^a$Heterogeneous glycosylation of human nectin-1 produces a broad mass distribution (~35–45 kDa); the mass of the major peak (~80%) is listed. $^c$Errors indicate the data range from at least two experiments. $^b$Sum of individual experimental masses. MW, molecular weight; ND, not determined.
with the results of previous in vitro binding assays, and, given that all combinations were tested here, confirm that these specific binding interactions represent the only strong heterophilic pairs in the nectin family. Notably, the SPR responses for heterophilic binding were always higher than those for homophilic binding at comparable protein concentrations, with homodimerization detectable only at the highest concentrations tested (Fig. 1 and Supplementary Fig. 1). We attempted to use quantitative kinetic and equilibrium analyses of SPR binding curves to determine the heterophilic nectin binding affinities, but the curves were a poor fit for the data, suggesting that this method was unsuitable for the determination of binding constants for these proteins (data not shown). Similar problems have been observed with other proteins that self-associate and are probably caused by the homodimerization of nectins within the immobilized and mobile phases competing with the measured binding interactions. Nonetheless, our semiquantitative data are consistent with previous studies showing that heterophilic binding is generally favored over homophilic binding.

To determine the stoichiometry of nectin heterophilic interactions in solution, we performed an equilibrium AUC analysis on a 1:1 mixture of human nectin-1 and nectin-3. Nectin monomers, homodimers and heterodimers are expected to be at equilibrium in the mixture, and the high affinity of the heterophilic interaction between nectin-1 and nectin-3 should result in an average apparent molecular mass in the AUC that approaches the actual molecular mass of the complex. Our analysis yielded an apparent molecular mass of 86.7 kDa, which corresponds closely to the molecular mass of a heterodimer (Table 1) and is suggestive of a 1:1 binding stoichiometry. An analysis of a mixture of nectin-1 and nectin-4 yielded similar results (Table 1). Together, our AUC and SPR data show that purified nectin ectodomains in solution form homophilic and heterophilic dimers with a pattern of activity and specificity that is consistent with the adhesive binding behavior observed in cellular studies.

**Homodimer structures of nectin-1–nectin-4 and Necl-5**

The overall structure of the nectin extracellular region was revealed in a recent structural study of a bacterially expressed, nonglycosylated full ectodomain region of nectin-1 (ref. 20). Researchers from the study identified a dimer interface between partner D1 domains, which they interpreted to represent a cis dimer putatively formed between nectins emanating from the same cell surface, but they identified no candidate trans interface. To help identify the trans adhesive interface, we determined the crystal structures of full ectodomains or adhesive fragments of human nectin-1 (D1–D3), mouse nectin-2 (D1–D2, two crystal forms), human nectin-3 (D1–D3), human nectin-4 (D1–D2) and human Necl-5 (D1–D3). The crystallographic statistics are summarized in Table 2. Each of these proteins has a similar overall structure, with an approximately linear arrangement of tandem immunoglobulin-like domains, and all structures except that of nectin-4 have N-linked glycosylation sites that are added in mammalian processing and are distributed among the D1, D2 and D3 domains, with no single glycan site conserved in all family members (Fig. 2).

For each nectin structure, we found a stereotyped homodimer arrangement between the membrane-distal D1 domains (Fig. 2 and Table 2). This dimer is the same one that was previously identified as representing a cis interface. However, we provide evidence below that this dimer corresponds to the nectin adhesive interface, and its structural details should therefore shed light on the adhesive binding specificities of the nectins.

In each crystal structure, the nectin dimer is either exactly or approximately twofold symmetric, with the majority of the interprotomer contacts being centered near the membrane-distal apex of the D1 domain. Like other immunoglobulin variable-like domains, D1 is comprised of two β-sheets that are formed by the ABED and CC′′ FG strands (Fig. 3a). The nectin dimer is formed by symmetrical interactions between the CC′′ FG sheets of two partner D1 domains. The twofold dimer axis bisects the dimer midway through the paired partner C strands, which form the center of the dimer interface. The F and G strands interact primarily through a substructure containing the FG loop at the apex of D1 that interacts with a pocket formed by the C′ and C′′ strands of the partner molecule. The homodimers of nectin-1, nectin-2, nectin-3, nectin-4 and Necl-5 bury a total of 1,699 Å², 1,675 Å², 1,341 Å², 1,341 Å² and 1,254 Å² of interfacial surface area (polar and hydrophobic), respectively, and are stabilized by a combination of hydrophobic contacts, hydrogen bonds and salt bridges.

Some characteristic interactions are conserved in the dimer interfaces of all five nectin structures. These include intercalation of a phenylalanine in the FG loop (Phe129, Phe136, Phe153, Phe132 and Phe128 in nectin-1, nectin-2, nectin-3, nectin-4 and Necl-5, respectively) into a hydrophobic pocket formed by the C′ and C′′ strands of the partner molecule (Fig. 3b). The phenylalanine acceptor pocket is characterized by a conserved glycine (Gly86, Gly85, Gly110, Gly87 and Gly83 in nectin-1, nectin-2, nectin-3, nectin-4 and Necl-5, respectively) whose Cα forms the base of the pocket, accommodating the phenylalanine aromatic ring. Residues flanking this glycine residue have a hydrophobic or a partial hydrophobic character, as do the adjacent residues on the C′ strand, which complete the pocket structure. Notably, the residue preceding the conserved glycine (Met85, Phe84, Tyr109, Tyr86 and Gln82 in nectin-1, nectin-2, nectin-3, nectin-4 and Necl-5, respectively) which has a hydrophobic or a partially hydrophobic character in nectin-1–nectin-4, forms the
space group
Nectin-1 D1–D3, human
Nectin-2 D1–D2, mouse, form 1
Nectin-2 D1–D2, mouse, form 2
Nectin-3 D1–D2, human
Nectin-4 D1–D2, human
Necl-5 D1–D3, human

Cell dimensions

| Space group | P63 | P312 | P312 | P622 | C2221 | P622 |
|-------------|-----|------|------|------|-------|------|
| a, b, c (Å) | 106.3, 106.3, 334.6 | 68.8, 68.8, 159.8 | 117.0, 117.0, 158.5 | 131.9, 131.9, 247.5 | 86.0, 142.8, 341.8 | 139.1, 139.1, 274.0 |
| α, β, γ (°) | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 |
| Resolution (Å) | 40–3.20 | 40–2.54 | 40–3.35 | 40–3.93 | 20–3.5 | 40–3.6 |
| Rsym | 0.10 (0.51) | 0.06 (0.54) | 0.09 (0.54) | 0.07 (0.61) | 0.161 (0.57) | 0.11 (0.55) |
| I/σI | 14.2 (2.2) | 20.5 (3.9) | 18.7 (4.0) | 24.0 (3.2) | 7.8 (2.4) | 13.7 (2.5) |
| Completeness (%) | 99.0 (92.4) | 99.4 (99.7) | 99.2 (99.9) | 98.3 (99.1) | 99.4 (100.0) | 99.8 (99.9) |
| Redundancy | 6.0 (4.8) | 6.1 (6.2) | 5.6 (5.7) | 5.9 (6.1) | 5.2 (5.5) | 6.3 (5.7) |
| Completeness (%) | 99.0 (92.4) | 99.4 (99.7) | 99.2 (99.9) | 98.3 (99.1) | 99.4 (100.0) | 99.8 (99.9) |
| Redundancy | 6.0 (4.8) | 6.1 (6.2) | 5.6 (5.7) | 5.9 (6.1) | 5.2 (5.5) | 6.3 (5.7) |

Table 2 Data collection and refinement statistics

Notably, in nectin-1–nectin-4, a charged residue in the F strand is positioned in the middle of each homodimer interface (Glu125, Glu132, Lys149 and Arg128 in nectin-1, nectin-2, nectin-3 and nectin-4, respectively; Fig. 3b) facing its symmetry-related residue in the partner molecule. This would be expected to result in electrostatic repulsion, potentially destabilizing the interface. That nectin-1–nectin-4 bind through this interface indicates that other favorable interactions (described above) outweigh this repulsion. Necl-5 has a hydrophobic amino acid, Leu124, at this position, distinguishing it from the classical nectins.

We tested the importance of the structurally observed dimer interface for binding between nectin ectodomain fragments in solution using an F129D mutant of nectin-1 that contains a substitution of the conserved residue Phe129 with aspartic acid to disrupt the interface by the introduction of an unpaired negative charge. An equilibrium AUC analysis of the mutant protein revealed its homodimerization to be severely weakened compared to that of the wild-type protein (Table 1). In SPR assays, the nectin-1 F129D mutant supported neither homophilic binding of nectin-1 nor heterophilic binding of nectin-3 or nectin-4 (Fig. 1f). An analogous mutation, F136D, in an adhesive D1–D2 fragment of mouse nectin-2 also abolished homodimerization in AUC experiments (Table 1). We determined the crystal structure of this mutant, revealing it to be a well-folded protein that does not form the characteristic dimer in the crystal lattice (Supplementary Fig. 4). Together, the mutagenesis data suggest that both homodimerization and heterodimerization of nectin ectodomains in solution depend on the interface present in the crystal structures.

Structural features unique to each nectin dimer interface

Although the topology of the homodimer interfaces in the nectin ectodomain crystal structures and a number of core interactions are conserved (as described above), the nature of some of the interface residues differ between the nectins (Fig. 3b and Supplementary Figs. 2 and 3). These differences potentially account for the variation in nectin homodimerization affinities. Nectin-1 and nectin-2, which have the tightest homophilic binding in AUC (Table 1), bury more hydrophobic surface in their dimer interfaces (734 Å² and 835 Å², respectively) than do nectin-3 and nectin-4 (635 Å² and 680 Å², respectively), which bind more weakly. The majority of the hydrophobic contacts are conserved among the nectins and are centered around the phenylalanine docking region, but one residue in the C′ strand is hydrophobic in the tight binders and is hydrophilic in the weak binders, accounting for much of the difference in the total buried hydrophobic area (Leu90, Pro89, Gln114 and Ser90 in nectin-1, nectin-2,
nectin-3 and nectin-4, respectively). The differences in hydrophobic contacts probably account, at least in part, for the stronger homophilic binding of nectin-1 and nectin-2.

Although Necl-5 crystallized as a homodimer in an arrangement essentially identical to nectin-1–nectin-4, there was no measurable homophilic binding of Necl-5 in our AUC experiments (Table 1), indicating that it can accommodate the conserved homodimer geometry but with very weak affinity. Notably, we found no strongly unfavorable electrostatic interaction at the Necl-5 homodimer interface, and the F-strand residue that is charged in the nectins is leucine in Necl-5 (Leu124; Fig. 3b). However, there are very few favorable interactions in this interface, and the total buried hydrophobic surface is less than in other nectin homodimers (619 Å²). This difference arises primarily from polar residues in the C strand and C′C″ loop of Necl-5 that are hydrophobic in the nectins (Fig. 3b and Supplementary Fig. 3). These include Ser87, which is hydrophobic in nectin-1 and nectin-2, and Glu82, which has a hydrophobic or a partially hydrophobic character in the nectins and interacts closely with the docked phenylalanine residue (Fig. 3b and Supplementary Fig. 3). Limited hydrophobic interactions at the homophilic interface are probably a major factor underlying both the absence of Necl-5 homophilic binding and the weak homophilic binding of nectin-3 and nectin-4.

The structurally identified dimer mediates trans adhesion

Geometrically, the conserved nectin homodimer in the crystal structures could, in principle, represent either a cis or a trans interaction. However, as it is the only interface present in the structures of all five nectin family members reported here and in the previously reported structures of nectin-1 (refs. 20,34), human nectin-2 D1 (ref. 35) and Necl-5 D1–D2 (ref. 36), and as mutation of this interface abolishes nectin binding (Fig. 1 and Table 1), the most parsimonious interpretation is that it directly mediates adhesive trans interactions.

To determine experimentally whether the structurally conserved homodimer interface mediates trans interactions under physiological conditions, we assessed the binding of full-length nectin-2 in transfected A431D cells using immunofluorescence and chemical cross-linking. The α splice isoform of mouse nectin-2, with a cytoplasmic Flag-mCherry tag (Fig. 4a), localized exclusively at the cell–cell contacts of transfected A431D cells in a pattern that was indistinguishable from that of endogenous nectin-2 in these cells (Fig. 4b). A point mutation, F136D, which inactivated nectin-2 dimerization in biophysical and structural experiments (Fig. 1, Table 1 and Supplementary Fig. 4), severely diminished the recruitment of the mutant nectin to cell contacts without affecting the localization of endogenous nectin-2, showing that the dimerization interface we observed in the crystal structures is essential for the recruitment of nectin to cell–cell contacts (Fig. 4b).

We then used targeted cross-linking to detect formation of the dimer interface found in the crystal structures on the cell surface. On the basis of the structure of the homodimer, we introduced single cysteine substitutions at three positions in the D1 domain of mouse nectin-2 to allow cross-linking of the specific dimer configuration at cell junctions using sulphydryl-reactive cross-linkers: S83C in the C′C″ loop near the apex of the dimer, L72C in the C strand near the base of the dimer and, as a negative control, T49C in the B strand far from the dimerization interface (Fig. 4c). We chose the distances between the mutant cysteines in the homodimers of the S83C and L72C mutants to match short 1,8-bis(maleimido)diethylene glycol (BM(PEG)₂) (14.7 Å) and long BM(PEG)₃ (17.8 Å) cross-linkers, respectively. The subcellular localizations of all the cysteine mutants were identical to that of wild-type protein, showing that the mutations did not affect nectin binding (Supplementary Fig. 5).

We first examined whether the nectin dimer could be specifically detected using this method. We cross-linked cells expressing the cysteine mutants with BM(PEG)₂ and BM(PEG)₃. Western blotting of the cell lysates using Flag-specific antibody clearly showed that the cross-linking led to the formation of adducts of high molecular weight that corresponded in size to the nectin dimers (Fig. 4d). In agreement with the structural
predictions, the L72C and S83C mutants could be efficiently cross-linked, whereas the T49C mutant, located far from the dimer interface, could not. Furthermore, the long cross-linker BM(PEG)3 was markedly more efficient for the L72C mutant, whereas the shorter cross-linker was more efficient for the S83C mutant, consistent with their relative spacing in the homodimer structure. Thus, our cross-linking assay specifically detects the nectin dimer configuration present in the crystal structures. To further substantiate this conclusion, we introduced the F136D mutation into the S83C mutant. As we expected, this double mutant could not be cross-linked into dimers (Fig. 4d).

Specific cross-linking using differentially tagged nectin-2 allowed us to determine the cis or trans orientation of the dimer at cell contacts. We cocultured cells expressing the nectin-2 L72C mutant tagged with Flag-mCherry with cells expressing the same mutant tagged instead with Myc-mCherry (Fig. 4a). We cross-linked the coculture with BM(PEG)3 and then lysed the cross-linked cells with 0.5% SDS-containing buffer to completely disrupt noncovalent protein-protein interactions while leaving covalent cross-linked interactions intact. We then precipitated the Flag-tagged nectin-2 L72C mutant with Flag-specific antibody. Western blotting of the resulting precipitates

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**Figure 3** Structural details of nectin homophilic dimers. (a) Ribbon diagram showing the homodimer interface of human nectin-1 D1–D3 formed between the CC′C″ FG faces of partner D1 domains. Protomers are colored in blue and salmon. The side chain of Phe129 in the FG loop, which interacts closely with the partner C′C″ strands, is shown in sphere representation. (b) Structural details of the conserved homodimer interfaces of human nectin-1, mouse nectin-2, human nectin-3, human nectin-4 and human Necl-5. 'Top view' and 'bottom view' correspond to the orientations indicated in a. N-linked glycans close to, but not participating in, the nectin-2 interface are shown as spheres. Side chains of residues that contribute at least 10 Å² of buried surface area in the homodimers are shown as sticks. Intermolecular hydrogen bonds between side chain atoms are shown as magenta dashes, and intermolecular salt bridges are shown as green dashes. A highly conserved phenylalanine residue is boxed in green in each interface to aid with comparison. A structurally analogous residue that is charged in nectin-1–nectin-4 and symmetrically apposed in each dimer is boxed in red (see main text).
using Myc-specific antibody clearly showed a cross-linked dimer that incorporated both the Myc- and Flag-tagged forms of the nectin-2 L72C mutant (Fig. 4e). This complex can form only as a result of specific cross-linking of the nectin dimer in the trans orientation between apposed cells expressing the Myc- and Flag-tagged forms. Our targeted cross-linking experiments thus provide direct evidence that the crystallographically observed dimer that is common to all the nectin structures reported here can form in the trans orientation between nectins on apposed cell surfaces. These observations, together with the results from our biophysical, structural and mutagenesis studies, strongly support the identification of this interface as the adhesive binding site of nectins.

**DISCUSSION**

The biophysical, structural and biochemical experiments presented here identify a canonical trans adhesive interface that mediates the intercellular adhesion between nectins. This dimer, which is present in all six of the unrelated crystal lattices reported here, has dimensions and an orientation that are compatible with a cell-to-cell trans orientation at adhesive junctions (Fig. 2). The distances between the C termini for the full ectodomain dimers of nectin-1, nectin-3 and Necl-5 range between 160 Å and 228 Å (Fig. 2), owing to subtype-dependent variations in interdomain angles and dimer angles (Supplementary Fig. 6). Assuming an extended conformation for the membrane-proximal stalk regions that are present in full-length nectins but absent from the crystallized constructs, the predicted intermembrane distances are approximately 244 Å, 268 Å and 265 Å for nectin-1, nectin-3 and Necl-5, respectively, which is compatible with intermembrane spacings of between 150 Å and 300 Å observed by electron microscopy of adherens junctions.

We note that the trans binding interface that we identify here could additionally form in a cis orientation between nectins that are not engaged in adhesion. Indeed, a previous cross-linking study showed that mutations targeting the same interface could partially inhibit the formation of putative nectin-1 cis dimers on the surfaces of dissociated cells. It is currently unclear whether formation of the nectin adhesive interface in cis has any physiologically relevant role. For cadherins, their rigid calcium-bound ectodomain structures geometrically prevent adhesive binding from the ectodomains presented on the same cell surface. Relaxation of cadherin rigidity by the removal of calcium allows cis dimers to form by the adhesive interface. Neclin could rely on similar structural principles to favor trans over cis binding.

The adhesive dimer described here is the only interface that is conserved in all six crystal lattices of wild-type nectins. The absence of other conserved interfaces across multiple structures indirectly argues against a physiological role for other ectodomain-mediated interactions between nectins. We did not find a potential cis dimer interface between parallel D2 domains described in a previously reported crystal structure of nectin-1 (ref. 38) in our nectin-1 structure, and we did not find an analogous interface for any other nectins, suggesting that this did not represent a biologically relevant interface. Our six structures did not reveal any other common interfaces, including any that could geometrically function to cluster nectins in cis. Thus, nectin clustering at cell junctions is probably not driven by direct nectin-nectin ectodomain interactions.

**Figure 4** Targeted cross-linking of the nectin homodimer interface in transfected A431D cells. (a) Schematic showing the nectin-2 Flag-mCherry or Myc-mCherry constructs used in these studies. Each tag is inserted between residues Pro513 and Ser514 in the nectin-2 cytoplasmic domain before the afadin-binding site. TM, transmembrane region. (b) Surface localization of wild-type Nectin-2 Flag-mCherry (top) and the Nectin-2 F136D Flag-mCherry interface mutant (bottom) in transfected A431D cells. Shown are red mCherry fluorescence (left) and fluorescein isothiocyanate immunofluorescence staining for endogenous nectin-2 (right). Scale bars, 20 µm. (c) Positions of the cysteine substitutions (T49C, L72C and S83C, green spheres) used for targeted cross-linking are shown on a ribbon diagram of the mouse nectin-2 homodimer. L72C and S83C, but not T49C, single mutants are predicted to permit cross-linking of the dimer interface. The conserved interface residue Phe136 is shown as sticks. (d) Targeted cross-linking of nectin-2 dimers. Reducing SDS-PAGE and anti-Flag western blot analysis of lysates from confluent cultures of A431D cells expressing the T49C, L72C or S83C single mutants or the S83C F136D double mutant of nectin-2 Flag-mCherry are shown. Without cross-linking, all mutants migrate as monomer bands of ~100 kDa (M, left). Cross-linker added to the confluent culture and quenched before lysis stabilizes a dimer form (D, ~200 kDa) in L72C and S83C transfectants only (middle and right). (e) Cross-linking of a nectin-2 homodimer interface in a trans orientation. Total cell lysates (left) and anti-Flag immunoprecipitates (right) determined by SDS-PAGE and blotting against Flag or Myc in A431D cells expressing the Flag-tagged form of the L72C mutant that were cocultured with cells expressing a Myc-tagged version. In each blot, lane 1 represents Myc-Flag cocultures without cross-linker, lane 2 represents Myc-Flag cocultures with cross-linker, and lane 3 represents Myc monocultures with cross-linker. The trans dimer (D, right) was immunoprecipitated (IP) with anti-Flag and blotted with Myc-specific antibodies.
Atomic-level basis for nectin adhesive specificity

The crystal structures reported here depict homophilic adhesive interfaces, but mutagenesis data suggest that heterophilic adhesive interactions use the same interface region (Fig. 1). In light of this and of the high structural similarity among nectins, an analysis of nectin homodimer interfaces, for which all the structures are now available, can reveal the molecular basis of heterophilic specificity. Note that in this analysis, the sequence numbering refers to the human nectins to restrict the analysis of specificity to a single species.

Tight heterophilic binding between cognate nectin pairs (nectin-1 and nectin-3, nectin-1 and nectin-4 and nectin-2 and nectin-3; Fig. 1) probably arises primarily from compatibility with respect to electrostatic interactions. As described above, a potential repulsive electrostatic interaction is present in homodimers of nectin-1 through nectin-4 between charged residues in the F strand that are symmetrically paired in the dimer (for example, between paired Glu125 residues in nectin-1; Fig. 3b). The charge of the residue at this conserved position defines two subgroups within the nectin family: comprising nectin-1 and nectin-2, for which this charge is negative (Glu125 and Glu141 (mouse Glu132), respectively), and nectin-3 and nectin-4, for which the charge is positive (Lys149 and Arg128, respectively). In all tight nectin adhesive heterodimers (nectin-1 and nectin-3, nectin-1 and nectin-4 and nectin-2 and nectin-3), each partner belongs to a different subgroup, so these charged residues, which lie at the center

Figure 5 Analysis of the molecular basis of nectin binding specificity. (a, b) Schematic diagrams of heterophilic binding pairs (a) and nonbinding pairs of human nectins (b), each modeled on the homophilic crystal structures. β-strands are labeled and are shown as arrows. A different colored background denotes each β-strand (top) or the position and orientation of the contacting β-strands of the partner molecule (bottom). The chemical nature (hydrophobic, polar or charged) of the interfacial residues are color coded as defined in the graphical legend. Interactions are denoted by arrows and are also color coded. Residues involved in hydrophobic contacts are surrounded with a brown dashed line. Favorable electrostatic interactions are shown in green, and unfavorable electrostatic interactions, which are characteristic of nonbinding pairs, are shown in orange. Chemical mismatches between residues are indicated with dashed lines. Note that throughout the figure, the single-letter amino acid abbreviations are used.
of each stereotyped interface, are paired with an opposite charge, allowing highly favorable salt bridges to form (Fig. 5a). This is in contrast to homodimers, where identical paired charges are expected to repel (Supplementary Fig. 3). Additionally, interface maps in which putative heterodimers are modeled on homodimer structures suggest that large hydrophobic contacts form at these heterophilic interfaces (Fig. 5a). Together, charge compatibility and buried hydrophobic surface areas provide a simple explanation for the observed inter-subgroup binding preference of nectins.

An exception to this pattern, that nectin-2 and nectin-4 do not bind despite belonging to subgroups with different charges, can be explained by the properties of their interfacial residues. Modeling of the putative nectin-2 and nectin-4 heterodimer on homodimer structures (Fig. 5b) reveals a potential repulsive interaction between positively charged residues in the C′C″ loops (Lys88 in nectin-2 and Lys85 in nectin-4) that probably destabilizes the dimer (Fig. 5b). Additionally, hydrophobic residues (Ala143 and Leu67) stabilizing the nectin-2 homodimer and polar residues (Ser130 and Gln64) stabilizing the nectin-4 homodimer would be mismatched in a heterocomplex (Fig. 5b, dashes).

Heterodimers between members of the same charge subgroup are not observed7,17,21,22 (Fig. 1). These heterodimers would include a repulsive electrostatic interaction between F strands (Fig. 5b), as is present in homodimers (Fig. 3 and Supplementary Fig. 3). Whereas favorable homodimer interactions can probably overcome this destabilization (as described above), such interactions would be absent from intrasubgroup heterodimer pairings because of mismatches in the interface. Between nectin-3 and nectin-4, polar and nonpolar residues would be mismatched, including Val151 in nectin-3 and Ser130 in nectin-4 near the center of the interface (Fig. 5b, dashes). Nectin-1 and nectin-2 are similarly mismatched, and additional electrostatic repulsion probably occurs between Lys75 of nectin-1 and Arg151 of nectin-2 in the C′C″ loop (Fig. 5b).

Binding interactions of Necl-5

We found a heterophilic interaction between Necl-5 and nectin-3 in our SPR analysis, which is in agreement with previous observations7,21,24. Notably, the binding responses of Necl-5 were very weak as compared to other heterophilic interactions7,21,24 (Fig. 1). Weak heterophilic binding of Necl-5, like its weak homophilic binding, probably results from a low amount of hydrophobic buried surface area in the dimer caused by the lack of hydrophobic residues in the phenylalanine acceptor pocket (Fig. 5a). The specificity of Necl-5 for nectin-3 may be the result of hydrophobic interactions near the center of the interface between Leu124 in Necl-5 and Val151 in nectin-3 (Fig. 5a).

Adhesive binding strengths of nectins

The homophilic and heterophilic binding affinities of nectins may be important for understanding their role in morphogenesis. Strong homophilic binding of nectin-1 and nectin-2 (Table 1) suggests potential homophilic roles in vivo, but these roles have not yet been defined, probably because phenotypes related to homophilic adhesion may be masked by functional redundancy between nectin subtypes in knockout studies10,25–27.

The quantitative relationship between the homophilic and heterophilic binding affinities of nectins remains unclear because of unique problems in the quantification of heterophilic binding affinities using SPR for molecules that bind homophilically and heterophilically through the same interface32,33 (as described above). Previous nectin SPR studies would have also been subject to the same technical difficulties that we experienced here that lead to artifacts in the determination of heterophilic binding constants. Additionally, reported nanomolar Kd values for nectin-1 and nectin-3 heterodimerization7,22 were derived from assays using nectins artificially dimerized through fusion to IgFc or alkaline phosphatase expression tags39, which would be expected to lead to apparent Kd values that are substantially lower than those of the true monovalent interactions40,41. Nevertheless, the results of our semiquantitative SPR analysis (Fig. 1) support previous findings showing heterophilic binding interactions to be stronger than homophilic interactions7,17,20–23 and are consistent with suggested roles for nectins in driving cell-sorting processes through differential adhesion10.

Binding modes in the wider nectin family

The structures of the nectin-like proteins Necl-1 (ref. 42) and Necl-3 (ref. 43), which belong to the synCAM subgroup6, revealed prototypical homodimeric structures that deviate in topology from the nectin homodimers described here. Both of these proteins form dimers through the C′C″ FG sheet of D1, as is common in immunoglobulin superfamily adhesion molecules44–46, but the characteristic phenylalanine docking present in nectins is not found in the nectin-like homodimers42,43. Notably, a recently described crystal structure of a heterocomplex between Necl-5 D1 and the T cell immunoglobulin and ITIM domain (TIGIT) protein47 showed a dimer interface that was remarkably similar to the homodimer interface observed here, despite substantial sequence divergence between TIGIT and the nectins. The scope of biologically relevant interactions that are mediated between nectins and other proteins through this interface remains to be fully determined.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Protein Data Bank: Coordinates for human nectin-1 D1-3, mouse nectin-3 D1-3, human nectin-3 D1-4, human Necl-5 D1-3 and mouse F136D mutant nectin-2 D1-2 have been deposited with accession codes 4FMF, 4FMK, 4FNO, 4FOM, 4FRW, 4FQP and 4FS0, respectively.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

O.I.H., J.B. and X.J. determined and refined all crystal structures. O.I.H. produced all wild-type and mutant proteins. P.S.K. performed and analyzed the SPR experiments. G.A. performed and analyzed the AUC experiments. J.V. performed all bioinformatic analyses. S.H., R.B.T. and S.M.T. performed immunofluorescence and cross-linking studies. O.I.H., B.H. and L.S. designed experiments, analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

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A key molecule essential for structural organization of cell-cell junctions of polarized epithelia during embryogenesis.

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ONLINE METHODS

Protein expression and purification. Complementary DNA sequences encoding native signal peptides and extracellular regions (D1–D3) of human nectin-1 (Met1–Pro337), nectin-2 (Met1–Pro350), nectin-3 (Met1–Pro339, splice form 1), nectin-4 (Met1–Gln336) and Necl-5 (Met1–Pro334); mouse nectin-1 (Met1–Pro337), nectin-2 (Met1–Pro341, splice form α), nectin-3 (Met1–Thr401, splice form α) and nectin-4 (Met1–Gly338); and the D1–D2 fragments of mouse nectin-2 (Met1–Tyr250) and human nectin-4 (Met1–Ser243) followed by a C-terminal hexahistidine tag were inserted into the HindIII and NotI sites of the mammalian expression vector pCEP4 (Invitrogen). The mouse nectin-3 construct included additional residues following the D3 domain comprising part of the 'stalk' region between D3 and transmembrane region; all other constructs had the stalks omitted. The point mutations F129D in human nectin-1 D1–D3 and F136D in mouse nectin-2 D1–D2 were introduced using the QuickChange method (Stratagen). We introduced constructs into HEK 293F cells using Lipofectamine 2000 (Invitrogen) and maintained transfectants under continuous selection with hygromycin B (Mediatech). Secreted hexahistidine-tagged nectins were collected from 4 l of conditioned media by nickel–nitrilotriacetic acid (Ni-NTA) affinity chromatography in a batch procedure (3, 25 °C) and were further purified by anion and cation exchange and size exclusion chromatography using an Akta fast protein liquid chromatography system (GE Healthcare). Pure proteins were concentrated to 5–15 mg/ml in 150 mM NaCl, 10 mM Tris-Cl, pH 8.0, except for the nectin-4 proteins, for which the NaCl concentration was 50 mM, and D1–D2 fragments, for which the Tris-Cl buffer was replaced with 20 mM Bis-Tris, pH 6.0.

Sedimentation equilibrium analytical ultracentrifugation. AUC equilibrium experiments were performed at 25 °C using a Beckman XL-A/1 ultracentrifuge with a Ti60An rotor. Data were collected using UV absorbance at 280 nm. Proteins were analyzed immediately after purification to avoid freeze-thaw cycles. Samples were dialyzed in 150 mM NaCl and 10 mM Tris-Cl, pH 8.0, except for the nectin-4 proteins, for which the NaCl concentration was 50 mM, and D1–D2 fragments, for which the Tris-Cl buffer was replaced with 20 mM Bis-Tris, pH 6.0.

Cell culture, cross-linking and immunoprecipitation experiments. Transfection, growth and immunofluorescence microscopy of human A431D cells were performed as described elsewhere43. The plasmid pRC-mN2 encoding the α splice form of mouse nectin-2 was constructed by inserting the coding complementary DNA into pRC-CMV between the HindIII and XbaI sites. Myc-mCherry or Flag-mCherry tags were inserted between Pro513 and Ser514 of the 'stalk' region between D3 and transmembrane region; all other constructs had the stalks omitted. The point mutations F129D in human nectin-1 D1–D3, 6% (v/v) PEG 6000, 0.05 M cadmium sulfate, 0.2 M sodium acetate and 0.1 M MES, pH 6.0, with 15% 2,3-butanediol cryoprotectant for crystal form 2 of mouse nectin-2 D1–D2; 13% (w/v) PEG 3350, 0.42 M sodium isothiocyanate and 0.1 M MES, pH 6.0, with 15% 2,3-butanediol cryoprotectant for crystal form 2 of mouse nectin-2 D1–D2; 1 M lithium sulfate, 0.6 M ammonium sulfate and 0.1 M trisodium citrate, pH 5.5, with 30% (v/v) glycerol cryoprotectant for the mouse nectin-2 F136D mutant; 4.6 M ammonium acetate and 0.1 M sodium acetate, pH 4.6, with no additional cryoprotectant for human nectin-3 D1–D3; 3 M NaCl and 0.1 M Bis-Tris, pH 5.5, with 30% (v/v) D-trehalose cryoprotectant for human nectin-4 D1–D2; and 55% (v/v) tacsimate (Hampton Research) and 0.1 M bicine, pH 9.0, with an additional 10% (v/v) tacsimate as cryoprotectant for human Necl-5 D1–D3.

Data were collected from single crystals at 100 K using a wavelength of 0.979 Å at the Northeastern Collaborative Access Team beamline 24-ID-E at the Advanced Photon Source, Argonne National Laboratory, Argonne, Illinois, USA (for human nectin-1 D1–D3 and human nectin-4 D1–D2) and at the X4A and X4C beamlines of the National Synchrotron Light Source, Brookhaven National Laboratory (all other crystals). Data were processed using HKL2000 (ref. 49). Structures were solved by molecular replacement using Phaser with human nectin-1 (3ALP20), or regions thereof, as a search model for all structures except human nectin-5 D1–D3, for which Necl-5 D1–D2 (3EOW; ref. 36) was used. Structures were refined by iterative rounds of model building in Coot and automated refinement using Refmac52 including translation-libration-screw (TLS) refinement. Noncrystallographic symmetry restraints were used in the refinements of human nectin-1 and nectin-4 and mouse nectin-2 form 2. The Ramachandran plot statistics (percentage allowed/disallowed) were: 96.1/3.9% (nectin-1), 94.5/5.5% (nectin-2 form 1), 95.9/4.1% (nectin-2 form 2), 89.7/10.3% (nectin-3), 95.9/4.1% (nectin-4) and 94.5/4.5% (Necl-5). Data collection and refinement statistics are listed in Table 2. Crystals of the F136D mutant of mouse nectin-2 D1–D2, belonging to space group P622 (a = b = 59.9 Å, c = 210.1 Å), diffracted X-rays to 3.25 Å, with a I/σ(I) of 14.2 (3.6 in highest resolution shell), a completeness of 99.2% (93.2) and an Rmerge of 0.12 (0.35). The structure was solved by molecular replacement with Phaser using wild-type nectin-2 (form 1) as search model and locating a single molecule in the asymmetric unit (solvent content 46%) with a translation function Z-score of 17.1. Using restrained refinement in reflmac against 3,967 unique reflections, 1,701 protein and 50 carbohydrate atoms were refined. A single round of refinement yielded a partially refined structure with Rmerge/Rfree of 23.7%/33.6% (Supplementary Figure 4). This was a mutant structure that was determined only to assess whether the binding interface was ablated, further refinement was not performed.
cells were either solubilized directly in the SDS gel sample buffer or subjected to immunoprecipitation, for which cells were extracted with immunoprecipitation SDS buffer (0.5% SDS in 10 mM Tris-HCl, pH 7.4, with 0.5 mM 4-(2-amino-ethyl) benzenesulfonfyl fluoride). After solubilization, DNA in lysates was broken with a 28G Insulin Syringe, and the SDS buffer concentration was adjusted to 0.1% with standard 1% Triton X-100 immunoprecipitation buffer. Lysates were cleared by centrifugation (14,000g), and Flag-tagged nectin was precipitated using anti-Flag agarose (Sigma).

**Modeling of nectin heterodimers.** Models for all heterodimeric pairs of human nectins were built based on the homodimer crystal structures. For nectin-2, the human homodimer structure (3R0N) was used to restrict the analysis to one species. Orientations of the partner molecules in the homodimer were used to position two protomers in the heterodimer by structural alignment. Heterodimer models were minimally refined using constrained minimization in Charmm,[54] which consisted of 100 minimization steps with the steepest descent algorithm followed by 300 steps with the conjugate gradient algorithm, whereas Cα atoms were harmonically restrained to their position with a force constraint of 20 kcal mol\(^{-1}\) Å\(^{-2}\).

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