Nectin and afadin: novel organizers of intercellular junctions

Yoshimi Takai* and Hiroyuki Nakanishi
Department of Molecular Biology and Biochemistry, Osaka University Graduate School of Medicine/Faculty of Medicine, Suita 565-0871, Japan

*Author for correspondence (e-mail: ytkai@molbio.med.osaka-u.ac.jp)

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Summary
The cadherin superfamily plays key roles in intercellular adhesion. An emerging intercellular adhesion system, consisting of nectin and afadin, also has roles in organization of a variety of intercellular junctions either in cooperation with, or independently of, cadherin. Nectin is a Ca2+-independent immunoglobulin-like intercellular adhesion molecule, and afadin is a nectin- and actin-filament-binding protein that connects nectin to the actin cytoskeleton. This novel intercellular adhesion system has roles in the organization of E-cadherin-based adherens junctions and claudin-based tight junctions in epithelial cells. The adhesion system is furthermore involved in the formation of synapses in neurons and the organization of heterotypic junctions between Sertoli cells and spermatids in the testis.

Key words: Nectin, Afadin, Adherens junctions, Tight junctions, Puncta adherentia junctions, Sertoli-cell–spermatid junctions

Introduction
Cells in multicellular organisms recognize their neighboring cells, adhere to them and form intercellular junctions that play essential roles in various cellular processes, including morphogenesis, differentiation, proliferation and migration (for reviews, see Takeichi, 1991; Gumbiner, 1996; Vleminckx and Kemler, 1999; Angst et al., 2000; Tepass et al., 2000; Yagi and Takeichi, 2000). Intercellular junctions are generally associated with the actin cytoskeleton, and this strengthens intercellular adhesion. In polarized epithelial cells, intercellular adhesion is mediated through a junctional complex comprising tight junctions (TJs), adherens junctions (AJs) and desmosomes (DSs) (Farquhar and Palade, 1963) (Fig. 1A). These junctional structures are typically aligned from the apical to basal sides, although DSs are independently distributed in other areas. TJs are likely to serve as a barrier that prevents solutes and water from passing through the paracellular pathway and as a fence between the apical and basolateral plasma membranes in epithelial cells (for reviews, see Tsukita et al., 1999; Tsukita et al., 2001). AJs play key roles in the formation and maintenance of TJs and DSs and furthermore in concentration of many biologically active molecules, such as membrane receptors, signaling molecules and oncogenes (for reviews, see Tsukita et al., 1992; Aberle et al., 1996; Anastasiadis and Reynolds, 2000; Gumbiner, 2000; Nagafuchi, 2001). TJs also concentrate tumor suppressor proteins and cell polarity proteins (Tsukita et al., 1999; Tsukita et al., 2001). However, how these molecules localize there remains unclear. The formation and disruption of the junctional complex are dynamically regulated by many extracellular and intracellular signals, such as scatter factor/hepatocyte growth factor and Ras, Rac and Cdc42 small GTPases (Gumbiner, 1996; Gumbiner, 2000). Again, the mechanisms that dynamically organize the junctional complex are largely unknown.

Synapses are a specialized form of intercellular junction, and their specificity and plasticity provide neurons with the structural and functional basis for the formation of networks (Fig. 1B). At synapses, there are two types of junctions: synaptic junctions and puncta adherentia junctions (Peters et al., 1976). Synaptic junctions include presynaptic active zones, where synaptic vesicles and Ca2+ channels localize, and post synaptic densities (PSDs), where neurotransmitter receptors localize. Puncta adherentia junctions by contrast are ultrastructurally similar to the AJs of epithelial cells. The homotypic intercellular junctions are formed by homophilic interactions between cell adhesion molecules (CAMs). This is in contrast to cell-matrix AJs, which form through heterophilic interactions between CAMs and matrix proteins.

In addition to the homotypic intercellular junctions described above, heterotypic intercellular junctions also exist. These include those formed between differentiating germ cells and their supporter Sertoli cells in the seminiferous epithelium in the testis and between specialized sensory cells and supporting cells in sensory epithelia. The seminiferous epithelium of the testis contains two types of intercellular junction: Sertoli-cell–Sertoli-cell junctions and Sertoli-cell–spermatid junctions (for a review, see Russell and Griswold, 1993) (Fig. 1C). Sertoli cells constitute the single-layered epithelium and embrace and cultivate spermatogenic cells throughout their development (spermatogenesis). During the latter half of spermiogenesis, spermatids form prominent heterotypic intercellular junctions with Sertoli cells (Sertoli-cell–spermatid junctions), which are downregulated when spermatids are released as spermatozoa. Sertoli-cell–Sertoli cell junctions, by contrast, are homotypic and similar to those in epithelial cells.

We describe here an emerging intercellular adhesion system consisting of nectin, a Ca2+-independent immunoglobulin (Ig)-like CAM, and afadin, a nectin- and actin filament (F-actin)-

unknown.
binding protein that connects nectin to the actin cytoskeleton. This novel adhesion system plays roles in the organization of all of these homotypic, interneuronal and heterotypic junctions.

**Molecular structures of nectin and afadin**

Nectin comprises a family of at least four members, nectin-1, nectin-2, nectin-3 and nectin-4, all of which, except nectin-4, have two or three splice variants: nectin-1α, nectin-1β, nectin-1γ, nectin-2α, nectin-2β, nectin-3α, nectin-3β and nectin-3γ (Morrison and Racaniello, 1992; Aoki et al., 1994; Lopez et al., 1995; Eberlé et al., 1995; Cocchi et al., 1998; Takahashi et al., 1999; Satoh-Horikawa et al., 2000; Lopez et al., 2001; Reymond et al., 2001). All the members, except nectin-1γ, have an extracellular region containing three Ig-like domains, a single transmembrane region and a cytoplasmic region (Fig. 2). Nectin-1γ is a secreted protein lacking the transmembrane region. Nectin is conserved from humans to rodents. It is highly homologous to the human poliovirus receptor (PVR), which has also four splice variants: PVRα, PVRβ, PVRγ and PVRδ (Mendelson et al., 1989; Koike et al., 1990). PVRα and PVRδ have a single transmembrane region, whereas PVRβ and PVRγ lack this. All the nectin family members, except nectin-1β, nectin-1γ, nectin-3γ and nectin-4, have a conserved four residue motif (Glu/Ala-X-Tyr-Val) that binds the PDZ domain of afadin at their cytoplasmic C-termini (Mandai et al., 1997; Takahashi et al., 1999; Satoh-Horikawa et al., 2000; Reymond et al., 2001). Although nectin-4 lacks this conserved motif, it nevertheless binds the PDZ domain of afadin (Reymond et al., 2001).

Afadin has two splice variants: l-afadin and s-afadin (Mandai et al., 1997). l-Afadin, the larger splice variant, is a nectin- and F-actin-binding protein that has two Ras-association (RA) domains, a forhead-associated (FHA) domain, a DIL domain, a PDZ domain, three proline-rich (PR) domains and an F-actin-binding domain (Mandai et al., 1997; Takahashi et al., 1999). l-Afadin binds along the side of F-actin but not to the ends of F-actin, although it does not have cross-linking activity. s-Afadin, the smaller splice variant, lacks the F-actin-binding domain and the third proline-rich domain. Human s-afadin is identical to the gene product of AF-6, a gene that has been identified as an ALL-1 fusion partner involved in acute myeloid leukemias (Prasad et al., 1993). AF-6/s-afadin has been reported to bind directly to RYK, a receptor tyrosine kinase, and a subset of Eph receptor tyrosine kinases (Hock et al., 1998; Buchert et al., 1999; Halford et al., 2000), but Trivier et al. have recently shown that AF-6/s-afadin does not in fact bind to RYK (Trivier and Ganesan, 2002). AF-6/s-afadin has also been reported to interact with a deubiquitinating enzyme, Fam (Taya et al., 1998), but Chen et al. have found no genetic interaction between the *Drosophila* homologs of mammalian l-afadin and Fam during its eye development (Chen et al., 2000). Unless otherwise specified, afadin refers to l-afadin in this article.

Nectin-1, nectin-2 and nectin-3 are ubiquitously expressed in a variety of cells, including fibroblasts, epithelial cells and neurons (Morrison and Racaniello, 1992; Aoki et al., 1994; Lopez et al., 1995; Lopez et al., 1998; Eberlé et al., 1995; Aoki et al., 1997; Cocchi et al., 1998; Takahashi et al., 1999; Satoh-Horikawa et al., 2000; Reymond et al., 2000; Haarr et al., 2001; Mizoguchi et al., 2002). Nectin-2 and nectin-3 are also expressed in cells that lack cadherins, such as B cells and monocytes, and spermatids (Aoki et al., 1997; Lopez et al., 1998; Bouchard et al., 2000; Ozaki-Kuroda et al., 2002). Human nectin-4 is expressed...
mainly in the placenta (Reymond et al., 2001). l-Afadin is ubiquitously expressed, whereas s-afadin is mainly expressed in neural tissue, although it is expressed at low levels in various other tissues (Mandai et al., 1997; Boettner et al., 2000).

**Intercellular adhesion activity of nectins**

E-Cadherin functions as a Ca\(^{2+}\)-dependent CAM at AJs in epithelial cells (Takeichi, 1991; Gumbiner, 1996; Vlieghe and Kemler, 1999; Tepass et al., 2000; Angst et al., 2000; Yagi and Takeichi, 2000). It is a member of the cadherin superfamily, which consists of >80 members. E-Cadherin has an extracellular region containing five tandemly repeated domains (EC1-EC5), a single transmembrane region and a cytoplasmic region (Fig. 3A). In the current model for its intercellular adhesion activity, which is based on crystallographic analysis of the EC1 and EC2 domains of E-cadherin and N-cadherin and of the whole domains (EC1-EC5) of C-cadherin (Shapiro et al., 1995; Nagar et al., 1996; Tamura et al., 1998; Pertz et al., 1999; Boggon et al., 2002), cadherin first forms a cis-dimer, in which the monomers are aligned in a parallel orientation, and then a trans-dimer, in which cis-dimers from opposing cell surfaces interact in an anti-parallel orientation. The formation of the cis-dimer appears to be essential for the formation of the trans-dimer (Tamura et al., 1998; Pertz et al., 1999).

In contrast to cadherins, the intercellular adhesion activity of nectins is Ca\(^{2+}\) independent (Aoki et al., 1997; Takahashi et al., 1999; Miyahara et al., 2000; Satoh-Horikawa et al., 2000). However, in common with E-cadherin, it is likely that nectin first forms a cis-dimer and then a trans-dimer (Fig. 3B) (Lopez et al., 1998; Miyahara et al., 2000; Satoh-Horikawa et al., 2000; Momose et al., 2002). Analysis of point and truncated mutants of nectin reveals that the formation of a cis-dimer is essential for the formation of a trans-dimer, whereas the latter is not essential for formation of the former. Each nectin family member forms a homo-cis-dimer but not a hetero-cis-dimer (Satoh-Horikawa et al., 2000), although the splice variants

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**Fig. 2.** Molecular structures of nectin and afadin. TM, transmembrane region.

**Fig. 3.** Models for the intercellular adhesion activities of cadherin and nectin. (A) Cadherin. (B) Nectin.
nectin-2α and nectin-2β form a hetero-cis-dimer (Lopez et al., 1998). Each member also forms a homo-trans-dimer (Aoki et al., 1997; Lopez et al., 1998; Takahashi et al., 1999; Miyahara et al., 2000; Satoh-Horikawa et al., 2000; Reymond et al., 2001; Momose et al., 2002). Nectin-3, however, can form a hetero-trans-dimer with either nectin-1 or nectin-2, and these hetero-trans-dimers are much stronger than homo-trans-dimers (Satoh-Horikawa et al., 2000). Nectin-3 also forms a hetero-trans-dimer with PVR (Reymond et al., 2001), and nectin-4 forms a hetero-trans-dimer with nectin-1 (Reymond et al., 2001). However, nectin-1 and nectin-2 do not form a hetero-trans-dimer (Satoh-Horikawa et al., 2000; Reymond et al., 2001). In this respect, nectins differ from cadherins, which form mainly homo-trans-dimers (Takeichi, 1991; Gumbiner, 1996; Vlemincks and Kemler, 1999; Angst et al., 2000; Tepass et al., 2000; Yagi and Takeichi, 2000).

A mutant of nectin lacking the second Ig-like domain does not form a cis-dimer, indicating that the second Ig-like domain

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**Fig. 4.** Molecular basis of homotypic, interneuronal and heterotypic junctions. (A) AJs and TJs. At AJs, E-cadherin serves as an essential CAM. The cytoplasmic region binds β-catenin, which in turn binds α-catenin. α-Catenin is associated with the circumferential F-actin bundles directly and indirectly through vinculin and α-actinin. Nectin also functions as a CAM at AJs, but is more highly concentrated at AJs than E-cadherin. The cytoplasmic region binds afadin that is directly associated with the F-actin bundles. Afadin and α-catenin are associated with each other presumably through an unidentified molecule ‘X’. At TJs, claudin and JAM function as CAMs. Occludin is another transmembrane protein at TJs. The cytoplasmic regions of claudin, JAM and occludin bind ZO-1, ZO-2 and ZO-3. ZO-1 and ZO-2 are directly associated with F-actin and form a dimer with ZO-3. Afadin and ZO-1 may be associated with each other through an unidentified molecule ‘Y’. F-Actin and peripheral membrane proteins shown in the right-side cell are omitted in the left-side cell. (B) Synapses. At the synapses between mossy fiber terminals and pyramidal cell dendrites in the CA3 area of hippocampus, both synaptic junctions and puncta adherentia junctions are highly developed. At the puncta adherentia junctions, nectin-1 and nectin-3 localize asymmetrically at the presynaptic and postsynaptic sides, respectively. Afadin, N-cadherin and catenins localize symmetrically at the both sides. Synaptic junctions are associated with presynaptic active zones where synaptic vesicles, Ca2+ channels and many other components, such as bassoon, localize and with PSDs where neurotransmitter receptors localize. F-Actin shown in the postsynaptic side is omitted in the presynaptic side. (C) Sertoli-cell–spermatid junctions. Nectin-2 in Sertoli cells and nectin-3 at spermatids form a hetero-trans-dimer at Sertoli-cell–spermatid junctions. The F-actin bundles surround the spermatid heads like parallel rings (3D view). The nectin-based adhesive membrane microdomains show one-to-one linkage with each F-actin bundle. ES contains not only F-actin bundles but also a flattened cistern that is connected to microtubules.
is necessary for the formation of the cis-dimer (Momose et al., 2002). Nevertheless, a fragment of the first Ig-like domain can form a cis–dimer (Krummenacher et al., 2002). This domain is thus also likely to be involved in the formation of the cis-dimer (Fabre et al., 2002; Krummenacher et al., 2002). In addition, the first Ig-like domain is necessary for the formation of the trans-dimer (Aoki et al., 1997; Miyahara et al., 2000; Sakisaka et al., 2001; Reynmond et al., 2001; Momose et al., 2002; Krummenacher et al., 2002). The first Ig-like domains of nectin-3 and nectin-4 bind to the same region of the first Ig-like domain of nectin-1 to form the respective hetero-transdimers, although nectin-3 shows higher affinity than nectin-4 (Fabre et al., 2002). The function of the third Ig-like domain is currently unknown. Note that the interaction between nectin and afadin is not essential for the formation of the cis-dimer or the trans-dimer (Miyahara et al., 2000).

Roles of nectin and afadin in organization of AJs in fibroblasts

In cultured fibroblasts that lack TJs, such as L cells that stably express exogenous E-cadherin (EL cells), nectin-2 and afadin colocalize with E-cadherin at intercellular AJs (Mandai et al., 1997; Takahashi et al., 1999). Neither nectin-2 nor afadin is found at cell-matrix AJs (Mandai et al., 1997; Takahashi et al., 1999). Several lines of evidence indicate that the nectin-afadin and E-cadherin–catenin systems are physically and functionally associated and that the systems play cooperative roles in the organization of AJs (Fig. 4A). Studies using L cells that express exogenous full-length or mutant nectin-1 or nectin-2 and E-cadherin have revealed that nectin recruits β-catenin through α-catenin and E-cadherin through the α-catenin and β-catenin complex to nectin-based junctions (Tachibana et al., 2000). Moreover, a recombinant extracellular fragment of nectin-3 fused to the Fc domain of IgG (Nef-3) forms a trans-dimer with nectin-1 and thereby inhibits the formation of the nectin-1-based intercellular junctions in EL cells (Honda et al., 2003). This inhibits the formation of the E-cadherin-based intercellular junctions. Conversely, Nef-3 coated on microbeads first recruits the nectin-1–afadin complex and then the E-cadherin–catenin complex to bead-cell contact sites in EL cells that stably express exogenous nectin-1 (nectin-1-EL cells) (Honda et al., 2003). An extracellular fragment of E-cadherin fused to the Fc domain of IgG (Cef) coated on microbeads recruits not only the E-cadherin–catenin complex but also the nectin-1–afadin complex to the bead-cell contact sites in nectin-1-EL cells (Honda et al., 2003). Kinetically, the nectin-based junctions form first and their formation is followed by formation of the E-cadherin-based junctions.

How the nectin-afadin system is physically associated with the E-cadherin–catenin system is not known, but both afadin and α-catenin have been shown to be essential for the association of nectin and E-cadherin. The cytoplasmic tail of E-cadherin and β-catenin might be involved in, but is not essential for, this association. Afadin directly binds α-catenin in vitro, but this binding is not strong (Tachibana et al., 2000; Pokutta et al., 2002). The direct binding of these proteins may occur in vivo, but it is more likely that a post-translational modification(s) of either or both proteins and/or an unidentified molecule(s) are required for the binding of α-catenin to afadin (Tachibana et al., 2000; Pokutta et al., 2002). α-Catenin also binds to ZO-1 – an AJ component in fibroblasts (Itoh et al., 1991; Itoh et al., 1997; Imamura et al., 1999; Gumbiner, 2000; Nagafuchi, 2001). Furthermore, ZO-1 binds to afadin (Yamamoto et al., 1997). ZO-1 might thus be involved in the association of nectin and E-cadherin, but this possibility is unlikely given that the direct binding of afadin to ZO-1 has not been reproduced (Sakisaka et al., 1999; Yokoyama et al., 2001) and nectin recruits ZO-1 to the nectin-based junctions through afadin in an α-catenin-independent manner (Yokoyama et al., 2001). Ponsin, an afadin- and vinculin-binding protein (Mandai et al., 1999), and vinculin colocalize with nectin and afadin at intercellular AJs and furthermore localize to cell-matrix AJs (Mandai et al., 1999). Vinculin directly binds to α-catenin (Tsukita et al., 1992; Aberle et al., 1996; Gumbiner, 2000; Nagafuchi, 2001). Vinculin and ponsin might thus also be involved in the association of nectin and E-cadherin. However, ponsin forms a binary complex with either afadin or vinculin and does not form a ternary complex – at least in a cell-free assay system (Mandai et al., 1999). Ponsin and vinculin are not essential for the association of nectin and E-cadherin (Tachibana et al., 2000).

Roles of nectin and afadin in organization of the junctional complex in epithelial cells

In the absorptive epithelial cells of mouse small intestine, nectin-1, nectin-2, nectin-3 and afadin are highly concentrated at AJs and absent from TJs and DSs (Mandai et al., 1997; Takahashi et al., 1999; Satoh-Horikawa et al., 2000) (Fig. 4A). Immunoelectron microscopy reveals that nectin-2 is confined to AJs, under which F-actin bundles lie. This distribution pattern is different from that of E-cadherin, which, although concentrated at AJs, is widely distributed from the apical to the basal sides of the lateral membrane (Tsukita et al., 1992; Gumbiner, 1996). Neither nectin-2 nor afadin is found at cell-matrix AJs (Mandai et al., 1997; Takahashi et al., 1999; Sakisaka et al., 1999; Asakura et al., 1999).

Several lines of evidence indicate that, as in fibroblasts, the nectin-afadin and E-cadherin–catenin systems are physically and functionally associated in epithelial cells. Firstly, during the formation of the junctional complex involving AJs and TJs in Madin Darby canine kidney (MDCK) cells that stably express exogenous nectin-1 (nectin-1-MDCK cells), E-cadherin is recruited to the nectin-1-based junctions where afadin colocalizes (Honda et al., 2003). Moreover, Nef-3 inhibits the formation of nectin-1-based intercellular junctions and thereby the recruitment of E-cadherin, and this inhibits formation of E-cadherin-based AJs in wild-type and nectin-1-MDCK cells (Honda et al., 2003). In the latter, Nef-3 coated on microbeads first recruits the nectin-1–afadin complex and then the E-cadherin–catenin complex to the bead-cell contact sites (Honda et al., 2003).

Further evidence is provided by the gastric cancer cell line, HSC-39. These cells express E-cadherin but do not form intercellular junctions (Yanagihara et al., 1991). Overexpression of nectin-2 or nectin-3 leads to intercellular adhesion (Peng et al., 2002). In keratinocytes N-cadherin mutant that lacks the extracellular region acts as a dominant negative mutant and reduces the E-cadherin-based adhesion.
activity (Fujimori and Takeichi et al., 1993). Overexpression of nectin-3 reverses this inhibitory action of the N-cadherin mutant (Y. Tanaka, H. Nakanishi, S. Kakunaga et al., unpublished). Overexpressed nectin-3 recruits the N-cadherin mutant to the nectin-3-based junctions. The N-cadherin mutant probably associates with endogenous nectin and therefore prevents it from associating with endogenous E-cadherin.

Afadin-deficient mice show developmental defects at stages around gastrulation, including disorganization of the ectoderm, impaired migration of the mesoderm, and loss of somites and other structures that are derived from both the ectoderm and the mesoderm (Ikeda et al., 1999; Zhadanov et al., 1999). Furthermore, cystic embryoid bodies derived from afadin-deficient embryonic stem cells show disorganization of the ectoderm (Ikeda et al., 1999). In the ectoderm of afadin-deficient mice and embryoid bodies, the organization of AJs is highly impaired. Again, these results strongly implicate the nectin-afadin system in the organization of intercellular junctions in these tissues. Single-molecule image analysis of nectin-2 and E-cadherin in mouse mammary tumor (MTD-1A) cells indicates that nectin-2 moves more rapidly than E-cadherin on the free surface of the plasma membrane (Katsuno et al., manuscript in preparation). These results suggest that the nectin-afadin and E-cadherin–catenin systems cooperatively organize AJs. The molecular mechanism by which the nectin-afadin and E-cadherin–catenin systems associate in epithelial cells thus appears to be similar to that in fibroblasts but has not fully been elucidated.

At TJs, three CAMs have thus far been identified (Fig. 4A). Claudin functions as a Ca2+-independent CAM (Tsukita et al., 1999; Tsukita et al., 2001) and constitutes a superfamily consisting of over 20 members. Occludin is another transmembrane protein at TJs, but its function has not yet been established. Junctional adhesion molecule (JAM) represents a Ca2+-independent Ig-like CAM family of at least three members at TJs (Martin-Padura et al., 1998; Ozaki et al., 1999; Palmeri et al., 2000; Liang et al., 2002). Claudin and occludin have four transmembrane regions, whereas JAM has an extracellular region that has two Ig-like domains, a single transmembrane region and a cytoplasmic region. Claudin, occludin and JAM are linked to the actin cytoskeleton through peripheral membrane proteins, including ZO-1, ZO-2 and ZO-3 (Tsukita et al., 1999; Tsukita et al., 2001; Bazzoni et al., 2000; Ebnet et al., 2000). ZO-1 and ZO-2 are F-actin-binding proteins and form a dimer with ZO-3 (Tsukita et al., 1999; Tsukita et al., 2001). The association of these CAMs with the actin cytoskeleton has been proposed to strengthen the intercellular adhesion of TJs (Tsukita et al., 1999; Tsukita et al., 2001). Although the formation of TJs is dependent on the formation of E-cadherin-based AJs (Takeichi, 1991; Gumbiner, 1996; Vlemincks and Kemler, 1999), accumulating evidence suggests that the nectin-afadin system plays a role in the organization of TJs. During the formation of the junctional complex of AJs and TJs in nectin-1-MDCK cells, claudin, occludin and JAM are recruited to the apical side of the nectin-1-based junctions, where both afadin and ZO-1 colocalize (Fukuhara et al., 2002a; Fukuhara et al., 2002b). After they are recruited, ZO-1 translocates from the nectin-1-based junctions to their apical side. Recruitment of claudin, occludin and JAM is inhibited by Nef-3 (Fukuhara et al., 2002a; Fukuhara et al., 2002b). Nef-3 coated on microbeads recruits not only the nectin-1-afadin complex but also ZO-1 and JAM to the bead-cell contact sites in nectin-1-MDCK cells (Fukuhara et al., 2002a). Nectin-2 recruits both α-catenin and ZO-1 at the same time to the nectin-2-based intercellular adhesion sites in L cells that stably express exogenous nectin-2 (nectin-2-L cells) (Yokoyama et al., 2001). This recruitment requires afadin but not α-catenin, ponsin or vinculin. In the ectoderm of afadin-deficient mice and embryoid bodies, organization of not only AJs but also TJs is highly impaired (Ikeda et al., 1999). Furthermore, mutations in the nectin-1 gene are responsible for clef lip/palate ectodermal dysplasia – Margarita island ectodermal dysplasia and Zlotogora-Ogür syndrome – which is characterized by cleft lip/palate, syndactyly and ectodermal dysplasia (Suzuki et al., 2000; Sozen et al., 2001). These results suggest that the nectin-afadin system plays a role in the organization of TJs. It is unknown how the nectin-afadin system regulates the organization of TJs, but ZO-1, ZO-2 and ZO-3 are associated with the nectin-afadin system may play a role in recruiting JAM, claudin and occludin. It also remains unknown whether JAM is involved in the localization of claudin and occludin for organization of TJs.

At the initial stage of formation of AJs and TJs, primordial spot-like junctions first form at the tips of the cellular protrusions that radiate from adjacent cells (Yonemura et al., 1995; Adams et al., 1998; Vasioukhin et al., 2000). Components of the E-cadherin–catenin and nectin-afadin systems, ZO-1 and JAM colocalize to these spot-like junctions, at which neither claudin or occludin is concentrated (Ando-Akatsuka et al., 1996; Asakura et al., 1999; Sakisaka et al., 1999; Ebnet et al., 2001). The spot-like junctions begin to fuse to form short line-like junctions, at which claudin and occludin do accumulate. Although the precise timing of arrival of each component at the junctional complex during its formation is unclear, we can propose the following speculative model for formation of the junctional complex.

In our model, all CAMs, including nectin, E-cadherin, JAM, claudin and occludin, are diffusely distributed on the free surface of the plasma membranes of migrating cells (Fig. 5A). When the two migrating cells contact through protrusions such as filopodia and lamellipodia, nectin and E-cadherin separately form trans-dimers that form micro-clusters at intercellular contact sites. Because, kinetically, nectin forms micro-clusters more rapidly than E-cadherin, the nectin-based micro-clusters are mainly formed at the initial stage. The nectin-based microclusters then recruit E-cadherin, which results in the formation of a mixture of nectin- and E-cadherin-based microclusters. The nectin and E-cadherin molecules in these microclusters are associated through afadin and catenins that are linked to the actin cytoskeleton. E-Cadherin-based micro-clusters that form slowly and independently of the nectin-based microclusters rapidly recruit the nectin-afadin complex to form other primordial spot-like junctions. These primordial junctions fuse with each other to form short line-like junctions, which develop into more mature AJs. During the formation of AJs, JAM is first assembled at the apical side of AJs, and this is followed by the recruitment of claudin and occludin presumably through ZO-1, ZO-2 and ZO-3, which eventually leads to the establishment of claudin-based TJs (Fig. 5B).
Nectin and afadin

Roles of nectin and afadin in organization of synapses

At the synapses between mossy fiber terminals and pyramidal cell dendrites in the CA3 area of the hippocampus, both synaptic junctions and puncta adherentia junctions are highly developed and actively remodeled in an activity-dependent manner (Amaral and Dent, 1981). Nectin-1 and nectin-3 localize asymmetrically at the presynaptic and postsynaptic sides, respectively, of the plasma membranes of the puncta adherentia junctions and form hetero-trans-dimers that form microclusters at intercellular contact sites. The nectin-based microclusters are mainly formed at the initial stage. The nectin-based microclusters then recruit E-cadherin, which results in the formation of a mixture of nectin- and E-cadherin-based microclusters (primordial spot-like junctions). These primordial junctions fuse to form short line-like junctions, which develop into more matured AJs. Upper panel, transverse section view; lower panel, cross-section view. (B) Formation of TJs. During the formation of AJs, JAM is first assembled at the apical side, followed by the recruitment of claudin, which eventually leads to the establishment of TJs (cross-section view).

Fig. 5. A model for the role and mode of action of nectin in the formation of a junctional complex in epithelial cells. (A) Formation of AJs. When the two migrating cells contact through their protrusions, nectin and E-cadherin separately form trans-dimers that form microclusters at intercellular contact sites. The nectin-based microclusters are mainly formed at the initial stage. The nectin-based microclusters then recruit E-cadherin, which results in the formation of a mixture of nectin- and E-cadherin-based microclusters (primordial spot-like junctions). These primordial junctions fuse to form short line-like junctions, which develop into more matured AJs. Upper panel, transverse section view; lower panel, cross-section view. (B) Formation of TJs. During the formation of AJs, JAM is first assembled at the apical side, followed by the recruitment of claudin, which eventually leads to the establishment of TJs (cross-section view).

Inhibition of nectin-1- and nectin-3-based adhesion by an inhibitor of nectin-1 (gD) in cultured rat hippocampal neurons results in a decrease in size and a concomitant increase in the number of synapses (Mizoguchi et al., 2002). The exact mechanism by which this occurs remains to be clarified but reflects partial inhibition of the formation of the hetero-trans-dimer between nectin-1 and nectin-3, which may affect N-cadherin-mediated adhesion and eventually lead to formation of smaller synapses. Why the number of synapses increases is not known either but may be due to a failure of neurons to determine the proper positions of synapses or compensation for functionally less competent smaller synapses. Thus, it is likely that the formation of the hetero-trans-dimer between nectin-1 and nectin-3 plays an important role in the determination of the position and the size of synapses – in cooperation with the N-cadherin–catenin system. This role of the nectin-afadin system is consistent with the finding that mutations in the nectin-1 gene are responsible for cleft lip/palate ectodermal dysplasia – Zlotogora-Ogür syndrome – which is characterized by mental retardation in addition to ectodermal dysplasia (Suzuki et al., 2000).
Synapses are formed by the meeting of axons and dendrites during their maturation. At primitive synapses, synaptic junctions and puncta adherentia junctions are not morphologically differentiated, but during the maturation of synapses membrane domain specialization gradually occurs (Amaral and Dent, 1981). This neural membrane domain specialization appears to be similar to that found during formation of the junctional complex in epithelial cells with respect to the dynamic localization patterns of the junctional proteins. It is postulated that primordial junctions form first, followed by transport of the components of active zones on dense core vesicles and subsequent formation of active zones at the presynaptic side (for reviews, see Desbach et al., 2001; Ziv and Garner, 2001). At the postsynaptic side, the components of PSDs are assembled, and membrane receptors on vesicles are transported to this region. We imagine that, in synaptogenesis, the nectin-afadin unit first forms primordial junctions, and this is followed by the recruitment of the N-cadherin–catenin unit. The components of active zones would then be recruited to the primordial junctions to form active zones at the presynaptic side. At the postsynaptic side, the components of PSDs would be assembled, and membrane receptors would be recruited. The membrane domains, comprising synaptic junctions and puncta adherentia junctions, would then gradually become segregated, and this would be followed by maturation of synapses.

**Roles of nectin and afadin in organization of Sertoli-cell–spermatid junctions**

Sertoli cells form a unique type of F-actin-based junctional complex referred to as the ectoplasmic specialization (ES) (for reviews, see Russell and Griswold, 1993; Vogl et al., 2000). ES contains F-actin bundles, which are arranged at regular intervals beneath the plasma membrane, and a flattened cistern of the endoplasmic reticulum, which is connected to microtubules (Fig. 4C). This is supposed to function as a scaffold that stabilizes an adhesive domain in the plasma membrane of Sertoli cells. Vinculin is concentrated at Sertoli-cell–Sertoli-cell junctions and Sertoli-cell–spermatid junctions (Grove and Vogl, 1989; Pfieffer and Vogl, 1991). Since the ES is formed only in Sertoli cells, Sertoli-cell–spermatid junctions are asymmetric. Moreover, unlike at typical AJs, the existence of the cadherin-catenin system has been questioned at Sertoli-cell–spermatid junctions (Byers et al., 1991; Cyr et al., 1992; Anderson et al., 1994). Thus, little is known about the intercellular CAMs responsible for Sertoli-cell–spermatid junctions. In contrast to Sertoli-cell–spermatid junctions, Sertoli-cell–Sertoli-cell junctions are equipped with TJs as well as AJs, which serve as the ‘blood-testis’ barrier.

Nectin-2, nectin-3 and afadin colocalize with the F-actin that underlies Sertoli-cell–spermatid junctions (Ozaki-Kuroda et al., 2002) (Fig. 4C). Nectin-2 and nectin-3 reside specifically in Sertoli cells and spermatids, respectively, which suggests the formation of a hetero-trans-dimer from nectin-3 in spermatids and nectin-2 in Sertoli cells. The nectin-based adhesive membrane microdomains show one-to-one linkage with each F-actin bundle at Sertoli cell–spermatid junctions. Nectin-2 and afadin localize at Sertoli-cell–Sertoli-cell junctions. Nectin-2-deficient mice exhibit male-specific infertility and have defects in the later steps of sperm morphogenesis, including distorted nuclei and an abnormal distribution of mitochondria (Bouchard et al., 2000). In these mice, the structure of Sertoli-cell–spermatid junctions is severely impaired, and the localization of nectin-3 and afadin is disorganized, whereas Sertoli-cell–Sertoli-cell junctions are apparently normal (Ozaki-Kuroda et al., 2002). The loosened adhesion and the lack of an F-actin scaffold due to mislocalization of afadin at Sertoli-cell–spermatid junctions may act together to render the contact site weak and convoluted and also produce the drastic condensation of spermatid nuclei.

On the basis of these observations, we propose a model for the molecular organization of Sertoli-cell–spermatid junctions in which nectin-2 on the Sertoli cell membrane forms a hetero-trans-dimer with nectin-3 on the spermatid membrane to form discrete adhesive membrane domains (Fig. 4C). In this model, the cytoplasmic region of nectin-2 in Sertoli cells binds to afadin to connect F-actin bundles to the membrane. The localization of afadin in spermatids remains unknown. It is likely that Sertoli-cell–spermatid junctions rely largely on the nectin-afadin system, whereas Sertoli-cell–Sertoli-cell junctions are formed through cooperation of multiple intercellular adhesion systems.

**Role of nectin as a receptor for α-herpes viruses for entry and intercellular spreading**

Nectin-1 was originally isolated as one of the poliovirus-receptor-related proteins and named PRR-1 (Lopez et al., 1995). Nectin-2 was originally isolated as the murine homolog of human PVR (Morrison and Racaniello, 1992) but turned out to be another poliovirus-receptor-related protein and was named PRR-2 (Eberlé et al., 1995). Neither PRR-1 nor PRR-2 has thus far been shown to serve as a PVR. They were later shown to serve as receptors for α-herpes viruses, facilitating their entry and intercellular spreading, and renamed HveC and HveB, respectively (Cocchi et al., 1998; Cocchi et al., 2000; Geraghty et al., 1998; Warner et al., 1998; Sakisaka et al., 2001). It remains unknown whether nectin-3 and nectin-4 serve as receptors for viruses. Human nectin-1 allows entry of all α-herpes viruses tested so far, including herpes simplex virus (HSV) type 1, HSV type 2 and pseudorabies virus (Spear et al., 2000; Campadelli-Fiume et al., 2000). Human nectin-2 can mediate entry of a restricted number of α-herpes viruses (Spear et al., 2000; Campadelli-Fiume et al., 2000). The usual manifestations of HSV disease are mucocutaneous lesions. HSV disease establishes latent infection of neurons in sensory ganglia and causes recurrent lesions at the sites of primary infection. In HSV disease, the intercellular spreading significantly contributes to the pathogenesis. The underlying mechanisms for entry and intercellular spreading of HSVs through nectin-1 are not fully understood, but at least four viral glycoproteins, gD, gB, and the gH-gL heterodimer, participate in the entry of HSV-1 (Spear et al., 2000; Campadelli-Fiume et al., 2000). Recombinant gD binds to nectin-1 in host cells and inhibits HSV-1 infection, which indicates that gD serves as a viral component that specifically interacts with its cellular receptor, nectin-1. The first Ig-like domain of nectin-1 is sufficient for the binding of gD and entry of HSV-1, and the second and third Ig-like domains increase the efficiency of entry (Cocchi et al., 1998). The interaction of nectin-1 with
Nectin and afadin

afadin increases the efficiency of intercellular spreading, but not the entry, of HSV-1 (Sakisaka et al., 2001). The E-cadherin–catenin system increases efficiency of both entry and intercellular spreading of HSV-1, which provides an additional line of evidence that nectin and E-cadherin are physically and functionally associated (Sakisaka et al., 2001).

Conclusions and perspectives

We have described here the roles of nectin and afadin in the organization of a variety of intercellular junctions in cooperation with, or independently of, cadherin. However, the detailed molecular mechanisms underlying the connection between the nectin-afadin system and other intercellular adhesion systems have not yet been fully elucidated. There may be other unidentified molecules that connect these different intercellular adhesion units, or post-translational modifications, such as protein phosphorylation, of the connectors. Elucidation of the molecular linkage between the nectin-afadin and other systems is essential for our understanding of the whole picture of the intercellular junctions. The nectin-afadin system may be involved in the dynamic formation and disruption of various types of intercellular junction and in the concentration of many biologically active molecules at intercellular junctions. In addition, the role of the nectin-afadin system at Sertoli–cell–spermatid junctions raises the possibility that this system is involved in many other heterotypic junctions between different types of cell. Since the nectin-based junctions form more rapidly than cadherin-based junctions, the nectin-afadin system may play a role in cell recognition. Elucidation of these unresolved issues will give us deeper insights into the molecular linkage between intercellular junctions and various cell functions, such as morphogenesis, differentiation, proliferation and migration, and also into the molecular mechanisms that underlie many human diseases arising from junctional disorders, such as cancer and vascular and mental diseases.

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