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The adhesive binding site of cadherins revisited

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Abstract

Cadherins are single-pass transmembrane proteins that, through their homophilic specificity, function in selective cell adhesion and sorting. They have a modular structure that includes an ectodomain composed of tandem 'cadherin domains,' which have a β-sandwich topology similar to that of immunoglobulin domains. Some early experiments suggest that, for the 'classical' cadherins, the adhesive specificity is encoded in the membrane-distal amino-terminal cadherin domain. Here, we review these data, and present new data that supports this idea.

Keywords: Cadherin; Cell adhesion; Homophilic binding

1. Introduction

Cadherins are the primary transmembrane proteins responsible for selective cell adhesion in both vertebrate and invertebrate animals [1,2]. The adhesive preference of the known cadherins is homophilic: a cadherin of type X will bind to another cadherin of type X, but will generally not interact with one of type Y, although there are some known cases of cross-interaction. Cells that adhere to one another often express the same cadherin(s). Expression of different cadherins in adjacent groups of cells is an important component in the separation of cell layers during devel-

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opment. This has been beautifully illustrated by Takeichi and his collaborators, in one example, in the separation of the neural tube from the ectoderm of developing embryos [3].

Cadherins comprise a large superfamily of at least 50 different family members sharing similar structures but yet different adhesive specificities [2,4]. The fact that most of the cadherins are expressed in the CNS where they act in connecting functionally related neuronal groups, as well as in the formation and maintenance of synapses suggests the intriguing possibility of organizing the brain by differential cadherin expression. The idea of wiring the brain by cell specific expression of cadherin family members has been further substantiated by the recent discovery of 52 CNS specific, cadherin-like genes which are organized in an unusual genomic locus [4,5].

Thus, understanding the molecular basis of cadherin adhesive specificity is a question of central importance to cell, neural, and developmental biology. The structure of cadherin family proteins is now well established [6–9]. They typically comprise an extracellular domain composed of several repeated homologous domains (five for the 'classical' and desmosomal cadherins, six or seven for the protocadherins), a transmembrane segment, and cytoplasmic domains that are conserved among the individual cadherin families.

What is the configuration of cadherin homophilic binding? Which of the extracellular domains interact to form adhesive bonds between cells? These questions have been addressed, so far, for the 'classical' cadherins — those that were first discovered, which include N-, E-, P-, and R-cadherins. Evidence from both in vitro and in vivo studies suggest that *cis* interactions between cadherins on the same cell surface are necessary for *trans* interactions between cadherins on the surfaces of adjacent cells. There has been a great deal of investigation into the formation of *cis* dimers [7,9–11]. In this paper, however, we will concern ourselves only with the question of the formation of *trans* or adhesive dimers formed between cadherins on the surface of adjacent cells.

Several lines of circumstantial evidence suggest that, for 'classical' cadherins, adhesion is mediated primarily through the membrane-distal amino-terminal cadherin domain. (1) Antibodies raised against the N-terminal domain of a desmosomal cadherin specifically inhibited adhesion in cells expressing this protein [12]. (2) Inexact cleavage of the pro-domain at the N-terminus results in the loss of adhesive function for E-cadherin [13]. These mutant proteins are expressed at the cell surface, and the cleavage site is only four amino acids upstream from the native site. Nonetheless, they fail to function. (3) Some mutations in the N-terminal cadherin domain have been shown to abolish adhesive capability in a number of different cadherins [13–17]. And (4) experiments using beads coated with a recombinant fragment encoding the two N-terminal domains of cadherin-11 showed measurable adhesive function [18]. The intermembrane spacing observed by electron microscopy at cadherin-mediated junctions (reports range from $\sim$200 to 350 Å) are too inaccurate to uniquely determine the *trans* domain pairing [7].

More direct evidence for N-terminal adhesion also exists. First, electron microscopy studies of an E-cadherin/COMP (cartilage oligomeric matrix protein) fusion protein, in which E-cadherin ectodomains are fused to the pentamerization domain of COMP, shows tip-to-tip association between *cis* dimers [19]. This tip-to-tip association, which originates most likely from *trans* interactions, can be abolished by single amino acid mutations in the N-terminal cadherin domain (Pertz et al., 1999). Moreover, depleting the Ca^{2+} binding pocket between the N-terminal cadherin domains one and two abrogates *trans* interactions as well [20].

Perhaps the most definitive demonstration of the role of the N-terminal domain comes from 'domain swapping' experiments. Takeichi and his collaborators, in an early study, showed that the adhesive specificity of E- and P-cadherins could be switched simply by exchanging their N-terminal cadherin domains [14]. It seems that this experiment argues strongly against the presence of binding sites on other domains.

Here we present new evidence which supports the idea that 'classical' cadherin specificity resides in the amino-terminal cadherin domain. We show

(1) that exchanging the amino-terminal domains of N- and E-cadherins exchanges the adhesive specificities of these molecules; and (2) that an amino-terminal domain deletion of N-cadherin completely abolishes the adhesive capacity of this molecule.

2. Results

2.1. N-cadherin lacking the EC1 domain does not mediate cell adhesion

To determine whether the EC1 domain of N-cadherin is necessary for the adhesion mediated by this molecule, we made an expression construct in which the EC1 domain was deleted (NΔEC1). In this construct, the signal peptide was fused directly to the start of the EC2 domain.

We tested for expression of this protein in L-cells by Western blotting and immunolocalization. The NΔEC1 protein could be detected with antibodies directed against the EC2 domain, but was not detected by antibodies directed against EC1 (Fig. 1). The staining shows localization at the cell surface, with very little internal staining, indicating that the protein is successfully exported to the plasma membrane.

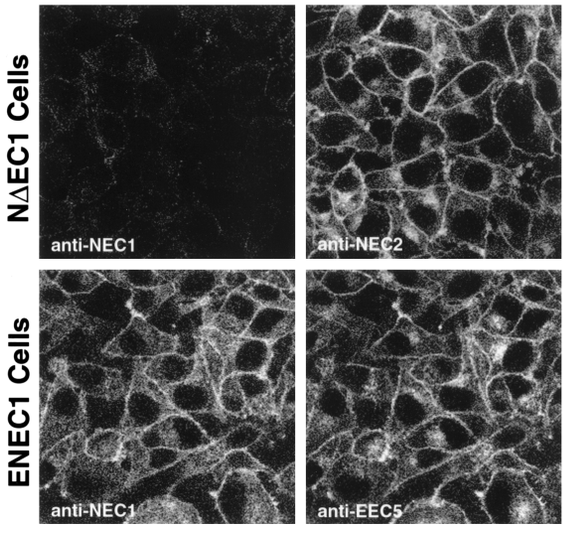

Fig. 1. Immunofluorescence staining of cultured L-cell transfectants. NΔEC1 transfectants show surface labeling with antibodies directed against the N-cadherin EC2 domain, but not the EC1 domain. ENEC1 expressors can be labeled with antibodies against N-cadherin EC1 or E-cadherin domains.

Cell aggregation assays (Fig. 2) with NΔEC1 transfected L-cells showed that these cells did not aggregate in either the presence or absence of calcium. Furthermore, when these cells (labeled with lipophilic dyes to distinguish them) were mixed with wild-type N-cadherin expressors, the wild-type expressors aggregated in a calcium-dependent way, but the NΔEC1 expressors failed to aggregate with them.

2.2. The adhesive specificity of N-cadherin is encoded in the EC1 domain

To determine whether the EC1 domain of N-cadherin is sufficient to provide specificity for the adhesive interaction between N-cadherin molecules on opposing cells, we produced a chimeric molecule, ENEC1, in which the EC1 domain from N-cadherin was substituted for the EC1 domain of E-cadherin. Immunofluorescent staining showed surface expression of this molecule, and could be detected either with antibodies directed against E-cadherin, or antibodies directed against the EC1 domain from N-cadherin.

Cell aggregation experiments (Fig. 3) show that ENEC1 is a calcium-dependent homophilic adhesion molecule, like native classical cadherins. Mixed-cell aggregation experiments show that ENEC1 expressing cells co-mingle and adhere with N-cadherin expressors, but sort out from E-cadherin expressors. Thus, ENEC1 appears to have the adhesive specificity of N-cadherin, and this specificity must therefore reside in the EC1 domain.

3. Discussion

The extracellular segments of cadherins are multi-modular. Thus, several possibilities for pair-wise interactions exist. For example, it might be possible that all domains are functional in adhesion, and line up in an antiparallel complete interdigitation such that domain pairs are formed

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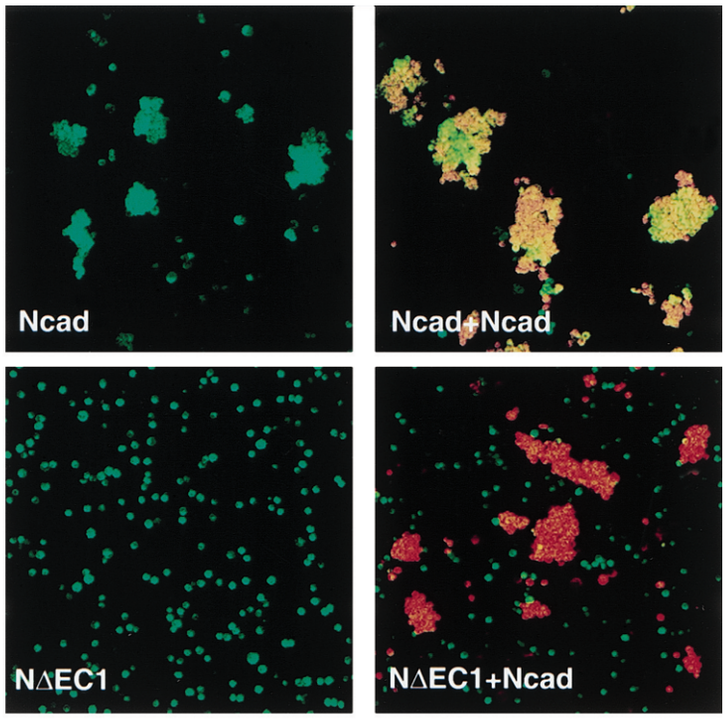

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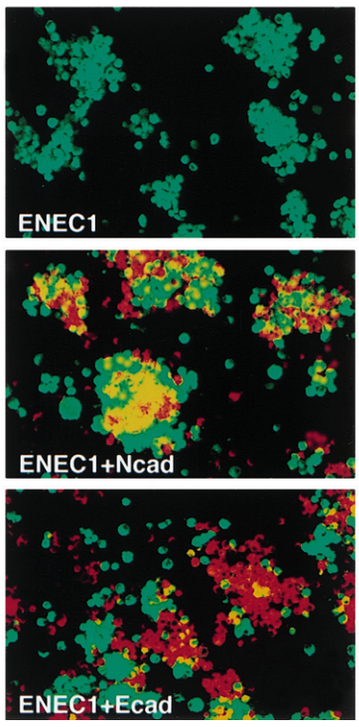

Legends to Figs. 2 and 3 on facing page.

between EC1 and EC5, EC2 and EC4, etc. Our data adds to the idea, however, that for the classical cadherins adhesion occurs mainly in the EC1 domain. While it might be suggested that other binding sites exist, this appears unlikely: If the binding sites for N-cadherin EC1 existed on N-cadherin EC5, for example, the ENEC1 chimera would probably be non-functional. Rather, ENEC1 exhibits adhesive functionality indistinguishable from N-cadherin, even though it contains only the EC1 domain from this molecule. These results are similar to those found previously for E- and P-cadherins [14]. This suggests to us that the adhesive function of classical cadherins is encoded primarily in their EC1 domains. Other EC domains may act, in part, as 'spacers' to achieve an optimal intercellular spacing.

4. Materials and methods

4.1. Antibodies

Polyclonal antibodies against the EC1 domain of N-cadherin and EC5 domain of E-cadherin were

4.2. cDNA constructs

cDNAs encoding full-length N-cadherin and E-cadherin were isolated by screening a mouse brain cDNA library. The coding region was sequenced and were identical to the published N-cadherin and E- [21,22]. Coding regions were inserted into the PcXN2 expression vector for protein expression in L-cells.

Deletion mutagenesis of N-cadherin EC1 domain was generated by removing a coding region from precursor peptide (710) to mature peptide (1256) by EcoNI and ligated. These mutations did not change the reading frame. To generate the chimeric construct ENEC1, the E-cadherin EC1 domain was substituted by the N-cadherin EC1 domain coding region. For this procedure, a 1.5-Kb fragment of E-cadherin containing signal peptide and mature peptide EC1–EC3 region was cut out and subcloned into XbaI and XhoI site of the pBluescript vector. A PCR fragment containing new Tth111I restriction site in E-cadherin was generated to match at the appropriate positions of N-cadherin, and ligated with a fragment from the N-cadherin EC1 domain, which was cut out by Tth111I, to yield the replacement of N-cadherin EC1 domain in E-cadherin. Finally, the whole chimeric cadherin cDNAs was cloned into the PcXN2 vector. All PCR products and ligation sites were sequenced using ABI automated DNA sequencing.

4.3. Cell culture and transfection

L cells were cultured in a humidified atmosphere of 10% CO_2 and in 90% DMEM containing 10% FBS. For obtaining single or double transfectants, the cells were transfected by using superfect (Qiagen Inc. Valencia, CA, USA) with 1 μg of each cadherin in PcXN2 vector in single or double transfection, and then cultured in selective medium containing 800 μg/ml of G418 (GIbco BR Gaithersburg, MD, USA). Resistant colonies were isolated and examined for N-cadherin, R-cadherin, E-cadherin expression by immunofluorescence staining, the positive cells were subcloned and used for further studies.

4.4. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde, delipidated in 100% methanol, permeabilized with 0.1% Triton X-100, and blocked with 5% normal goat serum in PBS. After incubation for 1 h at 37°C with primary anti-cadherin antibody, and then incubated for 30 min with fluorescent-conjugated secondary antibodies (Jackson Im-

Fig. 2. Cell aggregation experiments, all in the presence of calcium. N-cadherin expressors show self-aggregation, whereas NΔEC1 expressors do not self-aggregate or aggregate with wild-type expressors.

Fig. 3. Cell aggregation experiments, all in the presence of calcium. ENEC1 expressors self-aggregate, and aggregate with N-cadherin expressing cells. However, they sort-out from E-cadherin expressing cells.

munoResearch Laboratories, West Grove, PA, USA). Coverslips were then mounted and examined by confocal laser microscopy.

4.5. Aggregation assays

Monolayer cultures were treated with 0.01% trypsin in HCMF (HEPES-buffered Ca^{2+}, Mg^{2+}-free Hanks' Solution) supplemented with 1 mM $CaCl_2$ for 30 min at 37°C. The trypsinized cells were washed gently in HCMF containing calcium and 1% BSA at 4°C. This procedure can dissociate cell layers into single cells, leaving cadherins intact on the cell surface. After the cells were thoroughly dissociated, 5×10^5 cell per well were transferred to 24-well dishes for a final volume of 0.5 ml HCMF containing 1% BSA with or without 1 mM Ca^{2+}. The plates were rotated at 80 rev./min at 37°C for 45 min.

For analysis of mixed aggregation, cells expressing different cadherin constructs were labeled with different lipophilic dyes prior to mixing. We used DiI (1,1′-dioctadecyl-3,3,3′-tetramethylindocarbocyanine) and DiO (3,3-dioctadecyloxacargene, OR). Stock solutions of DiI were made by dissolving 2.5 mg DiI in 1 ml of 100% ethanol, and stocks of DiO were made by dissolving 2.5 mg DiO in 1 ml of 90% ethanol and 10% dimethylsulfoxide. These stock solutions were sonicated and filtered before use. To label cells with these dyes, they were incubated for 8 h at 37°C in serum-containing DMEM at final concentrations of 15 mg/ml and 30 mg/ml for DiI and DiO, respectively. The cells were washed extensively with HCMF containing calcium to prevent cross contamination of the dyes. After single cell suspensions were obtained as described above, 2.5×10^5 cells per well of each of two types were transferred to a 24-well dish. After rotating at 80 rev./min at 37°C for 45 min, 50 ml of the fixed aggregates were removed, placed on a slide, and covered with a coverslip and checked by confocal micoscropy.

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