N-glycosylation alters cadherin-mediated intercellular binding kinetics

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Summary

We present direct evidence that the N-glycosylation state of neural cadherin impacts the intrinsic kinetics of cadherin-mediated intercellular binding. Micropipette manipulation measurements quantified the effect of N-glycosylation mutations on intercellular binding dynamics. The wild-type protein exhibits a two-stage binding process in which a fast, initial binding step is followed by a short lag and second, slower transition to the final binding stage. Mutations that ablate N-glycosylation at three sites on the extracellular domains 2 and 3 of neural cadherin alter this kinetic fingerprint. Glycosylation does not affect the affinities between the adhesive N-terminal domains, but instead modulates additional cadherin interactions, which govern the dynamics of intercellular binding. These results, together with previous findings that these hypo-glycosylation mutations increase the prevalence of cis dimers on cell membranes, suggest a binding mechanism in which initial adhesion is followed by additional cadherin interactions, which enhance binding but are modulated by N-glycosylation. Given that oncogene expression drives specific changes in N-glycosylation, these results provide insight into possible mechanisms altering cadherin function during tumor progression.

Key words: Cadherins, Cell adhesion, Glycosylation, Kinetics, Micropipette

Introduction

Classical cadherins are crucial adhesion and architectural glycoproteins that maintain the integrity of intercellular junctions in all cohesive tissues (Gumbiner, 2005; Inoue et al., 2001). These proteins bind cadherins on adjacent cell surfaces to mediate cell–cell adhesion and trigger signaling cascades that control differentiation, proliferation and cell migration (Bhadriraju et al., 2007; Desai et al., 2009; Fournier et al., 2008; Li et al., 2010; Liu et al., 2006; Rowlands et al., 2004; Wong and Gumbiner, 2003). The protein architecture comprises an extracellular region, which embeds the adhesive function, a transmembrane domain and a cytoplasmic domain (Yap et al., 1997a). Five, tandemly arranged, β-barrel domains designated EC1–EC5, and numbered from the N-terminal domain, form the extracellular region (Fig. 1A,B). Cadherins form trans adhesive bonds between the EC1 domains of cadherins on apposing cells, and there is some experimental evidence that they also form lateral, cis interactions on the cell membrane (Briere et al., 1996; Kim et al., 2005; Klingelhöfer et al., 2002; Yap et al., 1998).

Cadherins are essential both for morphogenesis and for maintaining the mechanical integrity and barrier functions of adult tissues. Mutations that compromise adhesion activity or localization at cell membranes correlate with diseases such as cancer. In addition to mutations in the primary structure, aberrant N-glycosylation of cadherin extracellular domains is increasingly linked to the metastatic phenotype of several cancers (Geng et al., 2004; Guo et al., 2009; Jamal et al., 2009; Kitada et al., 2001; Nita-Lazar et al., 2009; Nita-Lazar et al., 2010; Pinho et al., 2011; Vagin et al., 2008). Epithelial cadherin (E-cadherin) has several N-glycosylation sites along the extracellular region. E-cadherin in breast tumors exhibits highly branched N-glycans on extracellular domains EC4 and EC5, and hyper-glycosylation at these sites destabilizes epithelial junctions and increases tumor progression (Pinho et al., 2009a; Pinho et al., 2009b; Pinho et al., 2011; Vagin et al., 2008). Conversely, hypo-glycosylation, achieved by ablating an N-glycosylation site in EC4 enhanced intercellular junction assembly, cytoskeletal remodeling at junctions and barrier integrity (Jamal et al., 2009; Liwosz et al., 2006; Nita-Lazar et al., 2010; Pinho et al., 2011). Neural cadherin (N-cad) contains eight N-glycosylation consensus sequences in the extracellular segment that are post-translationally modified (Guo et al., 2009). Ablating all eight sites increased intercellular junction stability, enhanced ERK signaling, and reduced cell migration in a scratch wound-healing assay (Guo et al., 2009). In contrast to E-cadherin, neural cadherin did not demonstrate significant N-glycosylation on domains EC4 and EC5 (Guo et al., 2009). Instead, eliminating three N-glycosylation sites at N273, N325 and N402 on extracellular domains EC2 and EC3 had the same functional impact as mutating all eight sites. Crosslinking studies further indicated that the loss of N-glycans on EC2–EC3 of N-cadherin increased the prevalence of dimers on cell surfaces, suggesting that N-glycosylation modulates cis dimerization (Guo et al., 2009).

There is strong in vivo evidence that specific alterations in the structures of N-glycans expressed on cell surface glycoproteins are common during the oncogenesis of epithelial cells. These changes are driven by the transcriptional regulation of specific
glycosyltransferases by oncogene expression (Buckhaults et al., 1997; Fernandes et al., 1991; Murata et al., 2000; Pierce, 2009; Seales et al., 2005; Zhao et al., 2008b). Collectively, several studies suggest that N-glycosylation alters cadherin-associated functions, but evidence that N-glycosylation directly affects intrinsic cadherin functions has been circumstantial. This is because glycosylation could also perturb trafficking to the cell membrane or alter cadherin interactions with other cell surface proteins such as growth factor receptors (Doherty and Walsh, 1996; Gumbiner, 1996; Perrais et al., 2007; Tzima et al., 2005; Williams et al., 2001a). Moreover, the mechanism by which N-glycosylation of the EC2–EC5 region could alter intercellular adhesion is not obvious because these glycosylation sites are distant in the sequence from the adhesive EC1 interface (Fig. 1A,B).

This study uses micropipette manipulation measurements (Fig. 2) to quantify the impact of N-glycosylation mutations on the intrinsic dynamics of N-cadherin-mediated intercellular binding. Micropipette measurements have been used to determine the two-dimensional affinities and dissociation rates of several membrane-bound adhesion protein receptors (Chesla et al., 2000; Chien et al., 2008; Huang et al., 2004; Huang et al., 2007; Jiang et al., 2011; Leckband, 2000; Long et al., 2001; Williams et al., 2001b; Zhang et al., 2005). Such measurements previously showed that C-cadherin-mediated intercellular binding kinetics exhibit a two-stage process in which an initial, fast EC1-dependent binding step is followed by a short lag period and a further, slower increase in binding probability (Chien et al., 2008). Here we describe the effect on this kinetic signature of ablating N-glycosylation sites in the EC2 and EC3 domains of neural cadherin (N-cad). The findings suggest a binding mechanism in which initial, trans cadherin ligation is followed by additional cadherin interactions, which augment binding but are modulated by N-glycosylation.

**Results**

**Wild-type human N-cadherin displays biphasic kinetics**

The micropipette measurements quantify the binding probability $P$, which is the number of detected cell binding events $n_b$ divided by the total number of cell–cell contacts $N_T$. The change in $P$ with cell–cell contact time thus reflects the time-dependent increase in intercellular bonds (Chesla et al., 1998). Figure 3 shows the binding kinetics measured between Chinese hamster ovary (CHO) cells that stably express wild-type human N-cadherin (hN-CHO WT) at 18 cadherins/μm$^2$ and red blood cells (RBCs) modified with immunoglobulin Fc-tagged chicken N-cadherin (ckN-cad–Fc) at a surface density of 10 cadherins/μm$^2$. The binding kinetics exhibits two distinct stages. The first stage is a fast, initial rise to a plateau $P_1$ at $\sim 0.41$ within the first 2 seconds. This is followed by a 2–5 second lag or induction phase, and then a slower, second rise to a higher binding probability at $P_2 \sim 0.55$. The second plateau $P_2$ is stable for contact times up to 30 seconds, and previous studies showed that this plateau persists for at least 45 seconds (Chien et al., 2008).

Previous studies similarly showed that C-cadherin, which is also a Type I classical cadherin, displays biphasic (two-stage) kinetics (Chien et al., 2008). Further studies with cadherin EC domain deletion mutants showed that EC1 is required for the first, fast binding step, and the full extracellular domain is needed for the lag and second binding step (Chien et al., 2008). The first, EC1-dependent binding step could therefore be described by a
simple binding mechanism for the formation of a trans adhesive dimer, \( R + L \rightarrow LR \).

The probabilistic kinetic rate equation describing this mechanism (Chesla et al., 1998) is:

\[
P(t) = 1 - e^{-A_m m_g k_f (1 - e^{-k_o t})}.
\]

where \( A_2 \) is the contact area (\( \mu m^2 \)), \( m_g \) and \( m_l \) are cadherin densities on the two cells (number/\( \mu m^2 \)), \( k_o \) is the two-dimensional affinity (\( \mu m^2 \)), and \( k_{off} \) is the dissociation rate (seconds\(^{-1}\)). Thus, the two-dimensional binding affinity and dissociation rates could be determined from fits of the kinetic data to Equation 1 (Chien et al., 2008). The solid line in Fig. 3 is the nonlinear least squares fit of the data to Equation 1. The best-fit affinity and dissociation rate are, respectively, \( 4.2 \pm 0.4 \times 10^{-4} \) \( \mu m^2 \) and \( 2.0 \pm 0.5 \) seconds\(^{-1}\) (Table 1). Importantly, Equation 1 does not describe the entire kinetic profile. Furthermore, the inclusion of a transient encounter complex (Harrison et al., 2010; Sivasankar et al., 2009) predicts an exponential rise to a single plateau, but it does not predict the two-stage kinetics shown here.

To identify data that correspond to the first, EC1-dependent step, we used a lack-of-fit test to parse the data into the two, distinct kinetic stages: namely the rise to \( P_1 \) and to \( P_2 \). This test compares the least squares residuals of the model (Equation 1) to the intrinsic variability of the data. When the test statistic exceeds the critical value for a given time point, then the model does not describe the data in question. To determine the dissociation rate and two-dimensional affinity for the initial step, Equation 1 was then fit to the maximum number of time points in each data set that did not fail the lack-of-fit test. This approach confirmed that Equation 1 does not describe the second increase in binding probability at longer times.

**N-glycosylation alters N-cadherin binding kinetics**

To determine the effect of N-glycosylation on N-cadherin-mediated intercellular binding kinetics, binding probability data were obtained with human N-cadherin mutants in which all eight of the N-glycosylation sites were mutated to Gln (hN-CHO MuALL) or sites 2, 3 and 4 (N273, N325 and N402) were mutated to Gln (hN-CHO Mu234) (Fig. 1A,B). In subsequent kinetic measurements, N-cadherin mutants were expressed on CHO cells at similar levels, as verified by western blot (Fig. 4). According to quantitative fluorescence-activated cell sorting (FACS) measurements, the expression levels were \( \sim 18 \) cadherins/\( \mu m^2 \).

The impact of hypo-glycosylation on the kinetics would depend on whether the lesser (limiting) protein ligand is the mutant expressed on CHO cells or the glycosylated ckN-cad–Fc bound to the RBCs. This is because the limiting reagent will dominate the kinetics. We therefore carried out measurements in which we varied the density of ckN-cad–Fc on the RBCs, so that the glycosylated protein was either less than (limiting) or in excess of the human N-cadherin (or mutant) on the CHO cells. The ckN-cad–Fc, which is expressed in mammalian cells, is glycosylated (Fig. 4B).

Fig. 5A shows the impact of the hypo-glycosylation mutations on binding kinetics, when the human N-cadherin mutants expressed on the CHO cells are limiting. The time course measured between hN-CHO WT (18 cadherin/\( \mu m^2 \)) and ckN-cad–Fc (37 cadherins/\( \mu m^2 \)) exhibits the biphasic kinetic signature (Fig. 5A). By contrast, ablating eight N-glycosylation sites (hN-CHO MuALL, \( \sim 18 \) cadherin/\( \mu m^2 \)) nearly eliminates the induction phase, and the binding probability rises smoothly to the higher probability plateau at \( P_2 \sim 0.63 \). The data do not exhibit a single exponential rise, indicating that at least two processes contribute to the overall kinetics.

Previous findings indicated that N-glycosylation sites in human N-cadherin domains EC2–EC3 (Fig. 1B) have the greatest effect on cadherin dimerization, cell migration and ERK signaling (Guo et al., 2009). The binding kinetics of the hN-CHO Mu234 variant is similar to that of hN-CHO MuALL, and the time course also exhibits a rapid rise to \( P_2 \sim 0.73 \) (Fig. 5A). The difference in amplitudes (\( P_1 \) and \( P_2 \)) measured with hN-CHO MuALL and hN-CHO Mu234 is due to the different ckN-cad–Fc densities used in the different measurements. Importantly, there is no significant induction phase observed with either of the hypoglycosylated N-cadherin mutants, when their densities are limiting. The altered kinetics relative to hN-CHO WT clearly

| Cell surface ligand | Ligand density (number/\( \mu m^2 \)) | ckN-cad–Fc (number/\( \mu m^2 \)) | Affinity (\( \mu m^2 \)) | Dissociation rate (second\(^{-1}\)) |
|---------------------|--------------------------------------|---------------------------------|--------------------------|-------------------------------|
| Human N-cadherin WT | 18                                   | 10                             | \( 4.2 \pm 0.4 \times 10^{-4} \) | \( 2.0 \pm 0.5 \)           |
| Human N-cadherin Mu2,3,4 | 18                                     | 7                             | \( 4.0 \pm 0.5 \times 10^{-4} \) | \( 1.4 \pm 0.6 \)           |
| Human N-cadherin MuALL | 18                                   | 7                             | \( 4.3 \pm 0.4 \times 10^{-4} \) | \( 2.2 \pm 0.5 \)           |
| Chicken N-cadherin  | 15                                   | 69                             | \( 1.9 \pm 0.3 \times 10^{-4} \) | \( 1.1 \pm 0.4 \)           |
shows that N-glycosylation on N-cadherin EC2–EC3 domains directly affects the intrinsic cadherin binding dynamics.

By contrast, when hypo-glycosylated N-cadherin is in excess and the glycosylated ckN-cad–Fc is the limiting ligand, the binding kinetics displays the induction phase. Fig. 5B shows the kinetic profiles measured with ckN-cad–Fc on the RBCs at 10 cadherins/μm² and 7 cadherins/μm². Measurements with hN-CHO WT and with the two, hypo-glycosylation mutants all exhibit the two-stage kinetic profile, as expected if the glycosylated ckN-cad-Fc densities were rate limiting and the glycans affect the kinetics. Interestingly, the best-fit, two-dimensional affinities for the first binding step, as measured with wild-type human N-cadherin and with the two glycosylation mutants (Table 1), are statistically similar, indicating that N-glycan removal does not affect EC1–EC1 binding.

Enzymatic deglycosylation of ckN-cad–Fc eliminates the induction phase
To test whether the lag phase observed in Fig. 3 and Fig. 5B is due to carbohydrates on ckN-cad–Fc, the glycans were trimmed enzymatically with exoglycosidases and EndoH under non-denaturing conditions. The ckN-cad–Fc extracellular domain contains six N-glycosylation sites, including sites at N278, N330 and N407 (Accession P10288), and therefore has a similar N-glycosylation profile to the human protein. Treatment with a mixture of EndoH, neuraminidase, β-galactosidase and PNGase reduced the apparent ckN-cad–Fc molecular mass by ∼15 kDa relative to the untreated protein (Fig. 4B). Under non-denaturing conditions, the enzymes are less efficient, so it is likely that some core glycans remain.

Consistent with expectations, the kinetics measured with ckN-cad–Fc did not exhibit the induction phase (Fig. 6A), at densities where ckN-cad–Fc is limiting (compare with Fig. 5B). In particular, the time course measured with enzymatically treated ckN-cad–Fc versus hN-CHO MuALL exhibits a smooth rise to a plateau at P∼0.6. The kinetic features measured with human N-cadherin mutants are similar qualitatively to the kinetics measured when the hypo-glycosylated mutant was limiting (compare with Fig. 5A).

The kinetics measured with hN-CHO WT and RBCs modified with treated (Fig. 6A, black circles) or untreated ckN-cad–Fc (Fig. 6B, black squares) clearly show that ckN-cad–Fc hypo-glycosylation eliminates the lag phase, and accelerates the rise to the higher binding probability P₂. Fig. 6B contrasts the kinetics measured with glycosylated proteins on both cells with those in which proteins on both cells are hypo-glycosylated. Similarly, the binding kinetics between identical RBCs modified with untreated ckN-cad–Fc on the RBCs (7 or 10 cadherins/μm²) and hN-CHO WT (black squares), hN-CHO ALL (white squares) or hN-CHO Mu234 (black circles) (∼18 cadherins/μm²) was measured with glycosylated N-cadherin on the cells in both cases. Although this could be due to slight differences in the intrinsic cadherin binding properties, the final binding...
probabilities measured with RBCs modified with either treated or untreated cK-cad–Fc (7 cadherins/μm²) were the same.

Discussion

The extent of N-glycosylation directly alters the intrinsic intercellular binding kinetics of neural cadherin. A large number of studies link aberrant glycosylation to altered cadherin-dependent cell functions, in the context of different cancers. Phenotypic changes include altered cell motility in scratch-wound healing assays, cytoskeleton reorganization, metastasis, ERK signaling and barrier function (Bajpai et al., 2008; Guo et al., 2009; Jamal et al., 2009; Liwosz et al., 2006; Pinho et al., 2009a; Pinho et al., 2011; Vagin et al., 2008; Zhao et al., 2008a). In the case of N-cadherin, the N-glycosylation sites at N273, N325 and N402 are highly conserved. Mutation of these sites ablates glycosylation and alters cell functions, but the loss of glycosylation does not affect cadherin trafficking to the membrane (Guo et al., 2009). Previous findings collectively suggested that glycosylation perturbs cadherin-dependent cell adhesion. The results of this study provide direct evidence that N-glycosylation alters the kinetics of N-cadherin-mediated intercellular bonds.

In the two-stage, kinetic time course, N-glycosylation modulates the duration of the induction (lag) phase and subsequent increase in binding probability, but not the intrinsic two-dimensional affinity of the EC1–EC1 bond. The glycosylation-independence of the affinity agrees with the similar measured affinities of N-cadherin EC1–EC2 fragments expressed in bacteria and in mammalian cells (Katsamba et al., 2009). The basis of N-glycosylation-dependent perturbations to cadherin therefore appears to differ from the sialylation of neural cell adhesion molecule, where the large excluded volume of polysialic acid blocks intercellular binding (Johnson et al., 2005). Instead, N-glycosylation mutations at N273A, N325A and N402A increased the prevalence of cis N-cadherin dimers on cell membranes (Guo et al., 2009). The latter result, together with the two-stage kinetics and its requirement for the full extracellular region (Chien et al., 2008), suggests a model in which initial adhesion facilitates additional cadherin interactions that enhance intercellular binding (Fig. 7). We speculate that the second, slower rise in binding probability is due to lateral association, which is in turn modulated by N-glycosylation.

Other observations are qualitatively consistent with the second step arising from lateral association. Increasing the cadherin density appears to reduce the induction phase, as expected for a binary reaction (see Fig. 5A,B). In a previous report, hexahistidine-tagged (monomeric) C-cadherin exhibited a longer induction time than Fc-tagged (dimeric) C-cadherin (Chien et al., 2008). Single-bond rupture studies and cell adhesion data suggest that cadherin dimers enhance adhesion relative to monomers (Zhang et al., 2009). Cross-linked dimers of C-cadherin extracellular domains also supported greater cell adhesion than monomers (Briehl et al., 1996).

Experimental evidence that cadherins dimerize on free cell membranes is mixed, however. At cell–cell junctions, cadherins form clusters involving many hundreds of proteins (Chtcheglova et al., 2010; Shewan et al., 2005; Yap et al., 1997a; Yap et al., 1997b). However, clustering requires active processes, and is unlikely driven by protein–protein interactions (Shewan et al., 2005). Chemical crosslinking did detect E-cadherin dimers on cells (Troyanovsky et al., 2003), but a similar approach failed to detect dimers of wild type N-cadherin (Guo et al., 2009), and
antibodies specific to monomeric or to dimeric cadherin detected only monomers on cells (Tsuji et al., 2007). Results of recent simulations, however, suggest that cell–cell adhesion might be required to overcome the energy barrier to lateral cadherin association (Wu et al., 2011). Entropy would contribute to this barrier, but glycosylation might also play a role. Steric interference by N-glycans could alter the free energy difference between monomers and dimers, and shift the dimer–monomer equilibrium, as suggested by crosslinking studies (Guo et al., 2009). Significant pre-dimerization would increase the first plateau $P_1$, but the effect of this initial equilibrium on subsequent kinetic steps depends on whether the clusters catalyze further oligomerization. Glycans could also create a kinetic barrier that would slow the dimerization rate, for example, by increasing the induction phase. Hypo-glycosylation through enzymatic treatment, point mutations or downregulation of glycosyltransferases would reduce the barrier and accelerate the rate of lateral association in intercellular gaps.

A similar mechanism is suggested by recent computer simulations of the adhesion-dependent coalescence of cadherin lattices between apposed surfaces (Harrison et al., 2011; Wu et al., 2010; Wu et al., 2011). The simulations constrained lateral cadherin association to mimic contacts in a crystal structure, where a frequently observed interface between EC1 and EC2 (Fig. 1B) is postulated to be the cis bond (Brasch et al., 2011). It is noteworthy that N273, which is one of the sites investigated in this study, lies at this postulated interface. The crystal structures were of deglycosylated proteins (Brasch et al., 2011), so it is not possible to visualize how the proposed cis interface might accommodate a large carbohydrate. The latter would likely generate a substantial steric barrier that could impede or disrupt the putative interaction.

Glycans on N-cadherin in melanoma lines have been characterized, but the glycans structure and molecular mass at each site is unknown and would vary with cell type and culture conditions. The 15 kDa shift in N-cadherin molecular mass following enzymatic treatment (Fig. 4B) gives an upper bound for expressed proteins in these studies. Additionally, prior characterization of the N-glycosylation mutants of human N-cadherin showed that each of the N273, N325 or N402 mutants alone did not generate the Mu234 or MuALL phenotype. Instead, the elimination of the induction phase required ablating all three N-glycosylation sites on both EC2 and EC3 domains, and suggests that the putative cis interface involves more extensive contacts between ectodomains – a possibility supported by the requirement of EC3 for the induction phase and second kinetic step (Chien et al., 2008).

Despite apparent parallels between our findings and the simulations (Harrison et al., 2011), there are important differences. First, the induction and second step in the kinetic profiles requires EC3 (Chien et al., 2008), which was not implicated in the structure-based model. Second, the effect of N325 glycosylation on the putative cis interface and junction organization is unknown. Third, it is unclear whether the sparse cadherin densities used in our studies would nucleate clusters, as depicted in the simulations. Although there might be different interpretations of the structural basis of these experimental observations, the results presented here are consistent with a process in which adhesion triggers subsequent changes in cadherin organization at cell–cell junctions that further enhances binding and is modulated by N-glycans.

This study focused on N-cadherin glycosylation, but E-cadherin hyper-glycosylation has been implicated in cadherin dysfunction in cancer (de Freitas Junior et al., 2011; Geng et al., 2004; Pinho et al., 2009a; Pinho et al., 2011; Zhao et al., 2008a). Although phenotypic changes of E-cadherin hypo-glycosylation (Jamal et al., 2009; Nita-Lazar et al., 2010) are qualitatively similar to those reported for N-cadherin (Guo et al., 2009), the glycosylation sites with the greatest impact on E-cadherin-based cell functions appear to be on membrane proximal domains EC4–EC5 (Liwoz et al., 2006). Despite the significance of these sites for E-cadherin, ablation of the same glycosylation sites on human N-cadherin did not alter ERK signaling or wound healing assays perceptibly (Guo et al., 2009). Studies have, however, documented altered N-linked glycan expression on specific receptors on tumor cells, driven by oncogene expression (Buckhaults et al., 1997; Fernandes et al., 1991; Murata et al., 2000; Pierce, 2009; Seales et al., 2005; Zhao et al., 2008b). It remains to be determined how the location and size of N-glycans on the extracellular domain, particularly those N-glycans whose structures change during oncogenesis, alter intrinsic cadherin function.

Finally, our use of human and chicken N-cadherin does not impact the conclusions of this study, primarily because we compared the effect of glycosylation on identical pair-wise interactions. The EC1 binding affinity between ckN-cad–Fc and chicken N-cadherin on CHO cells is slightly lower than that between ckN-cad–Fc and human N-cadherin (Table 1). Despite >90% sequence identity, small differences between the N-cadherins could affect binding in two ways. EC1 binding pocket differences could alter the affinities, or sequence variations could affect glycosylation. Chicken N-cadherin contains the consensus sequence for N-linked glycosylation at N278, N330, N407, N578, N628 and N657, and the first three sites correspond to the sites that were mutated in the human N-cadherin Mu234 variant. Similar N-glycosylation and our finding that glycosylation does not affect EC1-dependent affinities (Table 1) indicate that the affinity differences are due to binding site variations. However, neither the qualitative two-stage kinetic signature nor the induction phase depends on the EC1–EC1 affinity.

In summary, these results demonstrate that the N-glycosylation of neural cadherin directly impacts the intrinsic dynamics of cadherin-dependent intercellular binding. Glycosylation does not affect EC1-dependent affinities, but the two-stage kinetic fingerprint and prior crosslinking results support a binding mechanism in which initial, EC1-dependent trans binding is followed by additional cadherin interactions that enhance intercellular binding. N-glycans localized at three sites in the EC2–EC3 domains of N-cadherin alter the second step in the two-stage kinetic profile to modulate the intercellular binding dynamics.

Materials and Methods

Plasmids and cell lines

The extracellular region of chicken N-cadherin fused to a mouse Fc tag (ckN-cad–Fc) was previously stably expressed in CHO K1 cells (Prakash et al., 2006). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS (Invitrogen) and 0.4 mg/ml G418 (Sigma, St Louis, MO) as a selection marker. The ckN-cad–Fc construct was purified from cell supernatant using a Protein A affinity column (Bio-Rad) followed by gel filtration chromatography, as described previously (Chappuis-Flament et al., 2001; Prakash et al., 2006). CHO-K1 cells expressing the full-length human N-cadherin and glycosylation mutants of N-cadherin (Guo et al., 2009) were cultured in DMEM containing 10% FBS and 0.4 mg/ml G418. Quantitative flow cytometry and immunoblotting determined the cadherin expression levels.
Quantification of cadherin surface expression levels in CHO cells

Quantitative flow cytometry determined the density of cadherins expressed on the cell surface (Chesla et al., 1998; Chien et al., 2008). CHO-K1 cells expressing human N-cadherin were labeled with mouse anti-N-cadherin antibody and then with fluorescein-conjugated goat anti-mouse IgG antibody (Sigma). The antibody labeling was in phosphate buffered saline (PBS) [137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, 0.5 mM EDTA and 1% w/v bovine serum albumin (BSA) at pH 7.4]. An LSR II flow cytometer (BD Biosciences) measured the fluorescence intensities of labeled cells and of fluorescent bead standards (Zhang et al., 2005). The fluorescence calibration curve was obtained with calibrated FITC-labeled standard beads (Bangs Laboratories, Fishir, IN).

Immobilization of oriented cadherin extracellular domains on erythrocytes

Human whole blood was collected from healthy donors. The whole blood was stored in Vacutainers. The erythrocytes were isolated with Histopaque 1119 solution (Sigma) and then washed with ice-cold PBS and incubated with 1 mg/ml NHS-LC-biotin in PBS for 1 hour at room temperature with 0.9% w/v NaCl and resuspended in 12 ml EAS45 solution (2 mM CaCl2, 10 mM sodium phosphate dibasic, 55 mM NaCl, 10 mM glucose, and 20 mM Na2HPO4, at pH 8.0) (Dumaswala et al., 1996). The purified RBC suspension in EAS45 was maintained at 4°C for up to 3 weeks, after which the RBCs were treated with bleach and discarded.

Goat polyclonal anti-mouse IgG Fc-specific antibodies (Sigma) were covalently coupled to the CHO cells, activation (Gold and Fudenberg, 1967; Kolot, and Wick, 1977). Approximately 105 RBCs were washed five times with 0.85% w/v NaCl, and then resuspended in 250 μl of 0.85% NaCl with 1 μg of the antibody. A CRCl1 solution was diluted to concentrations below 0.01% w/v in 0.02 mM sodium acetate containing 0.85% w/v NaCl. Next, 250 μl of diluted CRCl1 solution was mixed with an equal volume of the antibody-modified cell and antibody mixture. After 5 minutes, 500 μl of ‘stop solution’ (PBS with 5 mM EDTA and 1% BSA) was added, and cells were washed twice with the ‘stop solution’. The labeled RBCs were stored in EAS45. The resulting density of antibodies immobilized to the RBC surface was variable, therefore the CRCl1 concentration was titrated to achieve the desired coverage. After labeling, a sample of 105 RBCs was taken and incubated with 8 μl of 1 mg/ml ckn-cad-Fc. The density of cadherin-Fc bound to the antibody-modified RBCs was determined by quantitative flow cytometry using the same antibody-labeling protocol that was used for the CHO cells expressing N-cadherin (Chesla et al., 1998; Chien et al., 2008).

Immunoblot analysis of cell surface expression levels

Subconfluent cells were washed and detached using 2 mM EDTA. Cells were centrifuged to remove debris. The concentrated supernatant was discarded as biological waste. The concentrated purified RBC suspension in EAS45 was maintained at 4°C for up to 3 weeks, after which the RBCs were treated with bleach and discarded.

Enzymatic de-glycosylation of cadherin–Fc constructs

To determine optimum conditions, 5 μl of antibody-modified RBCs was determined by quantitative flow cytometry using the same antibody-labeling protocol that was used for the CHO cells expressing N-cadherin (Chesla et al., 1998; Chien et al., 2008).

References

Bajpai, S., Correia, J., Feng, Y., Bautista, J. R., Sun, S., Longmore, G. D., Suriano, G. and Wirtz, D. (2008) alpha-Catenin mediates initial E-cadherin-dependent cell-cell recognition and subsequent bond strengthening. Proc. Natl. Acad. Sci. USA 105, 18331-18336.

Bladouirajl, K., Yang, M., Alom Ruiz, S., Pirone, D., Tan, J. and Chen, C. S. (2007). Triggering of E-cadherin: an integrin-like receptor? Exp. Cell Res. 313, 3616-3623.

Feist, J. L., Ahsen, G., Carnally, S. M., Henderson, R. M., Honig, B. and Shapiro, L. (2011). Structure and binding mechanism of vascular endothelial cadherin: a divergent classical cadherin. J. Mol. Biol. 408, 57-73.

Bromley, W. M., Yap, A. S. and Gumbiner, B. M. (1996). Lateral dimerization is controlled at the cell surface. Exp. Cell Res. 213, 56-58.

Buckhaulds, P., Chen, L., Fregien, N. and Pierce, M. (1997). Transcriptional regulation of N-acetylglucosaminyltransferase V by the srce oncogene. J. Biol. Chem. 272, 19575-19581.

Chapuis-Flament, S., Wong, E., Hicks, L. D., Kay, C. M. and Gumbiner, B. M. (2001). Multiple cadherin extracellular repeats mediate homophilic binding and adhesion. J. Cell Biol. 154, 231-242.

Chesla, S. E., Selvaraj, P. and Zhu, C. (1998). Measuring two-dimensional receptor-ligand binding kinetics by micropristie. Biophys. J. 75, 1553-1572.

Chesla, S. E., Li, P., Nagarajan, S., Selvaraj, P. and Zhu, C. (2000). The membrane anchor regulates ligand binding and two-dimensional kinetic rates and three-dimensional affinity of FcgammaRIII (CD16). J. Biol. Chem. 275, 10235-10246.

Chen, Y. H., Jiang, N., Li, F., Zhang, F., Zhu, C. and Leckband, D. (2008). Two stage cadherin kinetics require multiple extracellular domains but not the cytoplasmic region. J. Biol. Chem. 283, 1848-1856.

Chetrega, L. A., Wildling, L., Waschke, J., Drenckhahn, D. and Hinterdorfer, P. (2010). AFM functional imaging on vascular endothelial cells. J. Mol. Recognit. 23, 589-596.

Chotwick-Wierzbicka, D., Amoresano, A., Casbarra, A., Hoja-Lukowicz, D., Litynska, A. and Laider, P. (2004). The structure of the oligosaccharides of N-cadherin from human melanoma cell lines. Glyconcon. J. 20, 483-492.

de Freitas Junior, J. C. M., Silva, B. do R. A., de Souza, W. F., de Araujo, W. M., de Souza, L. C. S. F. W., da Silva, S. K., da Costa, B. and da Costa, B. (2011). Inhibition of N-linked glycosylation by tunicamycin induces E-cadherin-mediated cell-cell adhesion and inhibits cell proliferation in undifferentiated human colon cancer cells. Cancer Chemother. Pharmacol. 68, 227-235.

Desai, R. A., Gao, L., Raghavan, S., Liu, W. F. and Chen, C. S. (2009). Cell polarity triggered by cell-cell adhesion via E-cadherin. J. Cell Sci. 122, 905-911.

Doherty, P. and Walsh, F. S. (1996). CAM-COF receptor interactions: a model for axonal growth. Mol. Cell. Neurosci. 8, 99-111.

Dumaswala, U. J., Wilson, M. J., Jose, T. and Daleke, D. L. (1996). Glutamine and glutamine-containing storage media better maintain erythrocyte membrane physical properties. Blood 89, 697-704.

Evans, E., Leung, A., Heinrich, V. and Zhu, C. (2004). Mechanical switching and crossovers between two dissimilar pathways in a P-selectin adhesion bond. Proc. Natl. Acad. Sci. USA 101, 11281-11286.

Fernandes, B., Sagman, U., Auger, M., Demetrio, M. and Dennis, J. W. (1991). Beta 1-6-branched oligosaccharides as a marker of tumor progression in human breast and colon neoplasia. Cancer Res. 51, 718-723.
