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Kinetics of Adhesion Mediated by Extracellular Loops of Claudin-2 as Revealed by Single-Molecule Force Spectroscopy

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Claudins (Cldns) comprise a large family of important transmembrane proteins that localize at tight junctions where they play a central role in regulating paracellular transportation of solutes across epithelia. However, molecular interactions occurring between the extracellular domains of these proteins are poorly understood. Here, using atomic force microscopy, the adhesion strength and kinetic properties of the homophilic interactions between the two extracellular loops of Cldn2 (C2E1or C2E2) and full-length Cldn2 were characterized at the level of single molecule. Results show that while the first extracellular loop is sufficient for Cldn2/Cldn2 *trans*-interaction, the second extracellular loop does not interact with the full-length Cldn2, with the first extracellular loop, or with itself. Furthermore, within the range of loading rates probed (10^2–10^4 pN/s), dissociation of Cldn2/Cldn2 and C2E1/C2E1 complexes follows a two-step energy barrier model. The difference in activation energy for the inner and outer barriers of Cldn2/Cldn2 and C2E1/C2E1 dissociation was found to be 0.26 and 1.66 k_BT, respectively. Comparison of adhesion kinetics further revealed that Cldn2/Cldn2 dissociates at a much faster rate than C2E1/C2E1, indicating that the second extracellular loop probably has an antagonistic effect on the kinetic stability of Cldn2-mediated interactions. These results provide an insight into the importance of the first extracellular loop in *trans*-interaction of Cldn2-mediated adhesion.

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Introduction

Tight junctions (TJs) form a continuous belt of intercellular contacts in the apical region of epithelial monolayers. Their primary function is to regulate the paracellular transport of solutes across epithelia. The selective permeability of TJs largely results from a family of transmembrane proteins called claudins (Cldns).[1,2,3] With the use of dual pipette and cell aggregation assays, Cldn1 and Cldn2 were found to exhibit Ca^{2+}-independent adhesion activities.[4,5] However, little is known about the strength and kinetics of the interactions mediated by Cldns.

Structurally, Cldns consist of four transmembrane helices,[2] a short cytoplasmic N-terminal sequence, two extracellular loops, and an intracellular C-terminus

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Abbreviations used: Cldn, claudin; TJ, tight junction; AFM, atomic force microscopy; MC, Monte Carlo.

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that binds to cytoplasmic proteins through a PDZ motif.[6] The two extracellular loops of Cldns of adjacent cells *trans*-interact to form the paracellular TJ strands.

It has previously been shown that the first extracellular loop of Cldn2,[7,8] Cldn4,[8,9,10] Cldn5,[11] Cldn7,[12] Cldn8,[13] Cldn15,[9,14] Cldn16,[15] and Cldn19[16,17] confers charge-selective paracellular permeability to epithelial monolayers while the second extracellular loop acts as a receptor for a bacterial toxin for Cldn3[18] and Cldn4.[19] However, the interaction kinetics of the individual extracellular loops at the molecular level remains unclear. In this study, we have used single-molecule force spectroscopy to probe the molecular interactions between recombinant N-terminal glutathione *S*-transferase (GST)-tagged full-length human Cldn2 (GST-Cldn2) and the two extracellular loops of Cldn2 (GST-C2E1 and GST-C2E2) to gain an insight into the contribution of the individual extracellular loops to the overall adhesion kinetics.

Our results show that the first extracellular loop of Cldn2 is the major determinant of *trans*-interactions involving Cldn2. Dissociation of homophilic Cldn2/Cldn2 and C2E1/C2E1 complexes follow a two-energy-barrier model within the range of loading rates probed (10^2–10^4 pN/s). Comparison of interaction kinetics further revealed that Cldn2/Cldn2 dissociates at a much faster rate than C2E1/C2E1, implying that the second extracellular loop has an antagonistic effect on the kinetic stability of Cldn2-mediated adhesions.

Results

Measurement of Cldn2/Cldn2 and C2E1/C2E1 interaction forces

Trans-interactions between full-length human Cldn2 (Cldn2/Cldn2) or between first extracellular loops of Cldn2 (C2E1/C2E1) were characterized at the level of single molecule using atomic force microscopy (AFM) (Fig. 1).[20,21,22] The interaction was established by bringing GST-Cldn2 (or GST-C2E1) functionalized cantilever in close contact to a glass cover slip coated with GST-Cldn2 (or GST-C2E1) (see Materials and Methods). The functionalization of the tips and cover slips was confirmed using mouse anti-Cldn2 primary antibody (Abnova, Taiwan) and Alexa-488-labeled goat anti-mouse secondary antibody (Molecular Probes, Invitrogen). Confocal images showed that GST-Cldn2 was efficiently coupled to the AFM tips and cover slips (Fig. 2). In single-molecule force spectroscopy experiments, contact force and contact time are crucial for measuring discrete de-adhesion forces at molecular resolution.[21,23,24] When a contact force of 200 pN and contact time of 1 ms were used, <25% of the force–distance curves showed bond rupture events. On the basis of Poisson statistics,[25] the low frequency of these de-adhesion events ensured a >86% probability of the rupture being due to a single bond. Upon retraction of the cantilever, force as a function of pulling distance was recorded (Fig. 3a).[26] For each reproach velocity, hundreds of force–distance curves (n>500) were collected and analyzed to extract rupture force, F, and loading rate, r_f (Fig. 3b). The data obtained were subsequently pooled into histograms to analyze the frequency of adhesion events for different interactions (Table 1; Fig. 4). Binding was specific because adhesion frequency was significantly reduced in control experiments performed using AFM tips functionalized with only anti-GST antibody. Furthermore, blocking experiments performed using antibody specifically targeting the first extracellular loop of Cldn2 significantly reduced the binding frequency (Table 1; Fig. 4). The low frequency of interaction between Cldn1 and Cldn2 (Cldn1/Cldn2 or Cldn2/Cldn1, Table 1; Fig. 4) demonstrated that they do not *trans*-interact, which is consistent with previous findings.[27]

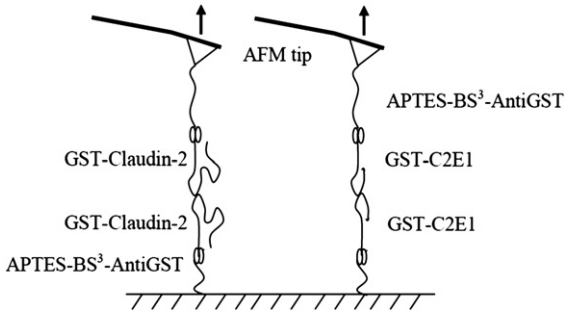

Fig. 1. Schematic of the AFM experimental setup. Recombinant GST-Cldn2 or GST-C2E1 was linked to the AFM tip or immobilized on glass cover slip using the linker APTES-BS^3-AntiGST (see Materials and Methods for details). GST-Cldn2 or GST-C2E1 immobilized on glass cover slip was probed using these functionalized tips. The arrow indicates the direction of pulling in the AFM experiment. GST, glutathione *S*-transferase; Cldn2, claudin-2; C2E1, first extracellular loop of Cldn2; APTES, 3-aminopropyltriethoxysilane; BS^3, bis(sulfosuccinimidyl) suberate; AntiGST, antibody targeting GST.

The first extracellular loop of Cldn2 is sufficient for promoting *trans*-interactions

Since Cldn2 consists of two extracellular loops (C2E1 and C2E2), the interactions observed between full-length Cldn2/Cldn2 could have resulted from interactions between two first extracellular loops (C2E1/C2E1), two second extracellular loops (C2E2/C2E2), or one first extracellular loop and another second extracellular loop (C2E1/C2E2) of apposing Cldn2 molecules. Histogram depicting the distribution of C2E1/C2E1 interaction forces demonstrated that the first extracellular loop can *trans*-interact with itself (C2E1_C2E1, Table 1; Fig. 4). Low adhesion frequency of C2E2/C2E2 interactions and significant reduction in Cldn2/Cldn2 interactions in the presence of an antibody targeting the first extracellular loop indicated that C2E2/C2E2 do not *trans*-interact (C2E2_C2E2 and Cldn2_Cldn2_Ab,

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