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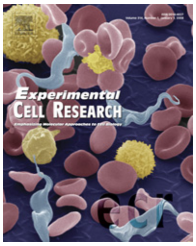

Research Article

Probing effects of pH change on dynamic response of Claudin-2 mediated adhesion using single molecule force spectroscopy

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ABSTRACT

Claudins belong to a large family of transmembrane proteins that localize at tight junctions (TJs) where they play a central role in regulating paracellular transport of solutes and nutrients across epithelial monolayers. Their ability to regulate the paracellular pathway is highly influenced by changes in extracellular pH. However, the effect of changes in pH on the strength and kinetics of claudin mediated adhesion is poorly understood. Using atomic force microscopy, we characterized the kinetic properties of homophilic *trans*-interactions between full length recombinant GST tagged Claudin-2 (Cldn2) under different pH conditions. In measurements covering three orders of magnitude change in force loading rate of 10^2–10^4 pN/s, the Cldn2/Cldn2 force spectrum (i.e., unbinding force versus loading rate) revealed a fast and a slow loading regime that characterized a steep inner activation barrier and a wide outer activation barrier throughout pH range of 4.5–8. Comparing to the neutral condition (pH 6.9), differences in the inner energy barriers for the dissociation of Cldn2/Cldn2 mediated interactions at acidic and alkaline environments were found to be $<0.65\ k_BT$, which is much lower than the outer dissociation energy barrier ($>1.37\ k_BT$). The relatively stable interaction of Cldn2/Cldn2 in neutral environment suggests that electrostatic interactions may contribute to the overall adhesion strength of Cldn2 interactions. Our results provide an insight into the changes in the inter-molecular forces and adhesion kinetics of Cldn2 mediated interactions in acidic, neutral and alkaline environments.

Introduction

Tight junctions (TJs) are the apical most constituents of the intercellular adhesion complex in epithelial monolayers. Their primary function is to regulate the paracellular transport of ions, solutes and water across epithelia. In addition, they interact with a variety of signaling and trafficking molecules to regulate cell differentiation, proliferation and polarity [1,2]. The selective permeability of TJs is largely determined by a protein family called claudins (Cldns) [3–5]. Although the contribution of

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Cldns to the charge selective permeability and ion homeostasis of epithelia is well established [6–16], details about the strength and adhesion kinetics of the interactions mediated by Cldns are being understood only recently [17].

Cldn2 was found to exhibit Ca^{2+}-independent adhesion activities in cell aggregation assays [18]. Expression of Cldn2 has been shown to induce cation-selective channels in TJs of epithelial cells [6]. Also, increased expression of Cldn2 has been shown to decrease transepithelial electrical resistance (TER) [19,20] while increasing the density of small TJ pores [21]. Furthermore, the knockdown of endogenous Cldn2 expression in MDCK cells using siRNA resulted in decreased Na^+ permeation and loss of cation selectivity [22]. In a more recent study, Cldn2 was shown to be critical for Vitamin D-dependent Ca^{2+} absorption between enterocytes [23]. Since it has been shown that the transport of several nutrients can be influenced by varying the extracellular pH [24–26], understanding the pH-associated changes of the adhesion kinetics mediated by Cldns will provide us a better perspective on how it regulates the paracellular transportation of solutes and intercellular adhesions. To address this question, we used single molecule force spectroscopy to investigate the molecular interactions between recombinant N-terminal glutathione S-transferase (GST) tagged full length human Cldn2 (GST-Cldn2) under different pH conditions (pH 4.5, 5.1, 6.9 and 8).

Our results show that dissociation of homophilic Cldn2/Cldn2 complexes follows a two-step energy barriers model within the pH range of 4.5–8 and loading rates of 10^2–10^4 pN/s. The energy landscape of the dissociations was found to be dynamically dependent on the changes in environmental pH. Comparison of adhesion kinetics further revealed that Cldn2/Cldn2 is relatively more stable in neutral solution (pH 6.9) when compared to acidic or alkaline environments, implying that electrostatic interactions may contribute to the adhesion strength of Cldn2 mediated adhesions.

Materials and methods

Protein immobilization and cantilever functionalization

Functionalization of AFM cantilevers was performed using methods described previously [27]. Soft silicon nitride tips (Vecco, Santa Barbara, CA) were UV irradiated for 15 min and incubated in a mixture of 30% H_2O_2/70% H_2SO_4 for 30 min. After washing thoroughly in ddH_2O, tips were dried and treated with a 4% solution of APTES (3-aminopropyltriethoxysilane, Sigma) in acetone for 3 min. They were then rinsed thrice in acetone and incubated in a solution of BS^3 (Bis (Sulfosuccinimidyl) suberate, 2 mg/ml, Pierce) for 30 min, followed by the incubation of anti-GST antibody (10 μg/ml, Invitrogen) for 2 h. The reaction was quenched using 1 M Tris buffer, followed by the incubation with recombinant full length GST-Cldn2 (10 μg/ml, Proteintech Group, Inc, USA) or GST-Cldn1 (10 μg/ml, Abnova, Taiwan) for 2 h. Unbound recombinant proteins were washed off with PBS. Tips were blocked in 1% BSA before experiments [27]. Recombinant GST-Cldn2 or GST-Cldn1 was immobilized on glass cover slips using the same procedure as described above. To confirm that GST-Cldn2 was efficiently linked to the silanized tips, primary mouse anti-Cldn2 antibody (Abnova, Taiwan) and Alexa 488-labeled goat anti-mouse secondary antibody (Molecular Probes, Invitrogen) were used to stain the GST-Cldn2-coupled tips. For control experiments, all steps were similar except that incubation of recombinant GST-Cldn2 proteins was omitted. For blocking experiments, tips and cover slips were incubated with antibody targeting the first extracellular loop of Cldn2 (10 μg/ml, Abnova, Taiwan) for 30 min. They were then washed to remove any unbound antibody before the experiments. For competition assays, interactions of GST-Cldn2/GST-Cldn2 were probed in the presence of GST-C2E1 (10 μg/ml, Abnova, Taiwan) (C2E1: first extracellular loop of Claudin-2, UUNP_065117UU, 29 a.a.–81 a.a.) or GST-C2E2 (10 μg/ml, Abnova, Taiwan) (C2E2: second extracellular loop of Claudin-2, UUNP_065117UU, 138 a.a.–163 a.a.) in PBS buffer.

Molecular force spectroscopy

Force curves were acquired on a MultiMode™ Picoforce™ AFM (Vecco, Santa Barbara, CA) coupled to an upright microscope at room temperature using a fluid cell. Cantilevers with a nominal spring constant of 0.01–0.03 N/m were used for obtaining force plots. Prior to obtaining force curves, the spring constant was determined using the thermal tune module. Target proteins (GST-Cldn2 or GST-Cldn1) immobilized on the glass cover slips were probed with cantilevers functionalized with recombinant proteins (GST-Cldn2 or GST-Cldn1) under different pH conditions (pH 4.5–8) in PBS buffer. Force plots were obtained at different reproach velocities (0.1–2 μm/s) and were analyzed for the magnitude of the rupture events and the apparent loading rate (defined as the slope of the retrace curve prior to the rupture event multiplied by the reproach velocity) using MATLAB version 7.1 (The MathWorks, Natick, MA.). Following Hanley et al. [28] and Panorchan et al. [29], rupture force measurements were partitioned by using binning windows of 50 pN/s for loading rates between 100 and 1000 pN/s and by binning windows of 500 pN/s for loading rates between 1000 and 10,000 pN/s. Each bin yields a mean force by Gaussian fitting. By plotting the mean force as a function of loading rate, the unstressed dissociation rate and reactive compliance characterizing Cldn2/Cldn2 interactions in different pH conditions were extracted (see Results section).

Results

Measurement of Cldn2/Cldn2 interaction forces

Trans-interactions between full length human Cldn2 (Cldn2/Cldn2) were characterized at the level of single molecule using atomic force microscopy (AFM) (Fig. 1) [28–30]. The interaction was established by bringing GST-Cldn2 functionalized cantilever in close contact to a glass cover slip coated with GST-Cldn2 under different pH conditions (pH 4.5–8) (see Materials and methods). To confirm that GST-Cldn2 was efficiently linked to the silanized tips, primary mouse anti-Cldn2 antibody and Alexa 488-labeled goat anti-mouse secondary antibody were used to stain the GST-Cldn2-coupled tips. Confocal images showed that GST-Cldn2 was efficiently coupled to the AFM tips and cover slips (Fig. 2).