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Cell surface N-glycans influence the level of functional E-cadherin at the cell–cell border

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ABSTRACT

E-cadherin is crucial for adhesion of cells to each other and thereby development and maintenance of tissue. While it is has been established that N-glycans inside the cell impact the level of E-cadherin at the cell surface of epithelial-derived cells, it is unclear whether N-glycans outside the cell control the clustering of E-cadherin at the cell–cell border. Here, we demonstrate reduction of N-glycans at the cell surface weakened the recruitment and retention of E-cadherin at the cell–cell border, and consequently reduced the strength of cell–cell interactions. We conclude that N-glycans at the cell surface are tightly linked to the placement of E-cadherin at the cell–cell border and thereby control E-cadherin mediated cell–cell adhesion.

1. Introduction

N-Glycosylation to newly synthesized membrane proteins is the most abundant protein co-translational modification in the lumen of the endoplasmic reticulum [1,2]. Further the process is critical for the survival of multicellular organisms as emphasized by the identification of congenital disorders of glycosylation (CDG) in humans [3,4], and mutant glycosylation mice [5–7] Given that the majority of membrane proteins are N-glycosylated, ascertaining the roles of glycans of membrane glycoproteins is instrumental in better understanding cellular interactions. Although it has been known that N-glycans inside the cell can impact the expression level of some membrane proteins by guiding their correct folding and assembly in the endoplasmic reticulum [8,9], it is unclear as to the roles of cell surface N-glycans in the spatial arrangement of glycosylated transmembrane proteins in the plasma membrane and thereby membrane architecture.

E-cadherin is an N-glycosylated transmembrane protein of epithelial cells. It has a major role in providing adhesive strength of cell–cell interactions through its action as the major component of adherens junctions, the structure which physically connects neighboring epithelial cells [10]. It has four utilized N-glycosylation sites and removal of these sites generates unstable protein that is not present at the cell surface [11]. Further, abrogation of the two sites closest to the N-terminus of E-cadherin alters calcium-dependent cell–cell adhesion, while vacancy of the site closest to the C-terminus has an immense influence on protein stability. More recently, it was shown that the N-glycans synthesized by cells impacts the clustering of E-cadherin glycoproteins at the cell–cell junction [12].

Loss of E-cadherin function in epithelial derived tumors results in tumor proliferation, thereby making tumor treatment much more difficult [13]. Additionally, different glycosylated forms of the E-cadherin protein can increase metastasis and progression of malignancy. For instance, E-cadherin expressed in cells that increase the number and degree of β1,6-GlcNAc branched N-glycans are more likely to strengthen tumor progression and metastasis while increases in E-cadherin with oligomannose N-glycans have tendencies to weaken these processes [14]. Our earlier studies show that E-cadherin glycoprotein molecules with oligomannose N-glycans localize to the cell–cell border to a greater extent than that with complex N-glycans, which also correlates with higher degree of cell–cell interactions in dissociation assays [12]. As such, our previous study reveals that specific N-glycans structures alter the spatial arrangement of E-cadherin in the plasma membrane.

In the present study, we show further that reducing the level of N-glycans at the cell surface lowers the recruitment and/or retention of E-cadherin at the cell–cell interface, and subsequently decreases the strength of cell–cell adhesion. Results of this study reveal a novel role of N-glycans at the cell surface in localizing E-cadherin to the cell–cell interface and thus the development and maintenance of tissue.

2. Materials and methods

2.1. Cell culture and transfections

Parental Pro-5 CHO cells (American Type Culture Collection, Manassas, VA, USA) were cultured in MEM Alpha Media (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum, 50 U/mL penicillin and 50 µg/mL streptomycin (Gemini BioProducts, West Sacramento, CA, USA) at 37 °C under 5% CO_2. Pools of stable transfected E-cadherin-GFP cells were developed as described elsewhere [12]. E-cadherin-GFP expression vector (GenBank: L08599) purchased from Addgene (Cambridge, MA, USA) was used for the studies.

2.2. Treatment of cells with PNGase F

For microscopy measurements and isolation of proteins, non-confluent monolayers of E-cadherin transfected Pro-5 cells were treated with PNGase F (50 U/mL) prior to and after cell–cell contacts. The selection for the amount of the PNGase F was based on adding various amounts (0–100 U/mL) of the enzyme to cultured cells in serum free medium as shown in results section. In brief, cells were seeded on culture dishes for 1 h and 25 h prior to treatment, respectively. The reaction occurred in serum free medium for 4 h at 37 °C under 5% CO_2. Treated cells were then cultured in complete medium for about 20 h and up to 1 h if cell–cell contacts were unestablished and established, respectively. For dissociation assays, confluent monolayers of nontransfected and E-cadherin transfected Pro-5 cells were treated with and without PNGase F under similar conditions.

2.3. Whole cell lysate and total membrane preparations

E-cadherin transfected cells treated or not treated with PNGase F were washed with PBS on the cell dish. RIPA buffer (PBS, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) plus protease inhibitor cocktail set III (EMD Biosciences, San Diega, CA, USA) was added to the washed cells and then cells were placed into a microcentrifuge tube. Cells were then drawn up in a 1 mL syringe with a 21 gauge needle to shear the DNA. After incubating the sample on ice for 30–60 min, it was centrifuged at $15,000 \times g$ for 20 min at 4 °C. Supernatants were collected and protein levels quantified using a modified Lowry method [15]. An aliquot of samples was resuspended in SDS–PAGE sample buffer for lectin blotting while

the remainder was stored at −20 °C. Total membrane isolations and PNGase F digestion reactions of total membranes were performed as previously described [12,16].

2.4. SDS-gels and glycoprotein blotting methods

SDS–PAGE was performed using 10% polyacrylamide gels with 5% stacking gels for lectin blots and Western blots. Whole cell lysate samples (5–25 µg) were loaded on SDS-gels and electrophoresed for 1.7 h at 20 mA. In some cases, gels were fixed and stained with Coomassie Brilliant Blue R-250. Separated proteins were transferred to membranes for 3 h at 250 mA and membranes were developed as we previously described for lectin and Western blots [12]. Biotinylated L-PHA (Vector Labs, Burlingame, CA, USA) was used to detect complex N-glycans. In all cases, the blots were reproduced 3 times.

2.5. Total internal reflection fluorescence microscopy

E-cadherin transfected Pro-5 cells were seeded onto 35 mm poly-L-lysine coated glass bottom dishes (MatTek, Ashland, MA, USA). The total incubation time of cells on cell culture dishes were 25–26 h or 29.5–31.5 h for PNGase F treatment prior to and after cell–cell contacts, respectively. Total internal reflection fluorescence (TIRF), differential interference contrast (DIC), and wide-field images of the cells were captured with an ORCA R2 deep cooled mono CCD camera connected to an Olympus IX-71 microscope (Olympus, Center Valley, PA, USA) equipped with a Apo 60× 1.45 objective as described [12,17]. An argon laser beam of wavelength 488 nm was used for excitation of EGFP. Exposure time of 1000 ms was utilized for data analysis of TIRF images. Detection settings were kept constant. Cell^TIRF Control 1.1 and Metamorph for Olympus Basic software controlled the shutters, filters, camera, and data acquisition. Image J software was used to measure the fluorescence intensity signal for the relative amount of protein in the membrane patch, including that at and away from the cell–cell border [12]. Experiments were performed on at least three different days.

2.6. Dissociation assay

Dissociation assays, along with analysis, were conducted in a similar manner as previously described [12,17]. Equal amounts of cells were seeded onto 35 mm dishes as we previously described. Confluent monolayers of nontransfected and E-cadherin transfected Pro-5 cells were treated with and without PNGase F after cell–cell contact, as described in Section 2.2. After treatment, confluent cell monolayers were washed twice with MEM. Cells were then removed by one complete rotation with a cell scraper and cell clumps were dissociated by pipetting seven times. Images (30 fields/dish) were obtained on an Olympus IX 50 microscope using a 10× objective. Particles are cell aggregates with more than five cells, and the number and area of the particles were measured. Data was collected from 3 different experiments.

2.7. Data analysis

Mean fluorescence intensity was determined using Image J software. Kodak image gel logic 100 imaging system was used to capture images of Coomassie stained SDS-gels, and lectin and Western blots. Image files were then transferred to Adobe Photoshop for generation of figures. Origin 7.5 was used for graphics and statistics. Data is presented as the mean ± S.E. where *n* denotes the number of fluorescence intensity ratios or cell clusters. The unpaired Student's *t*-test and one-way ANOVA with Bonferroni adjustment was utilized for comparing 2 populations and more than 2
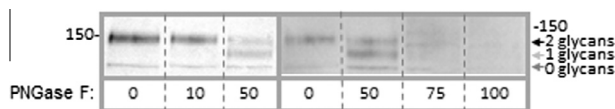

Fig. 1. Western blots of the Kv3.1b protein from live cells treated with PNGase F. Total membranes from Kv3.1b transfected Pro-5 CHO cells were treated with different amounts of PNGase F after formation of cell–cell contacts, as indicated. Black and light gray arrows point to the fully (2 glycans) and partially glycosylated (1 glycan) Kv3.1b proteins, respectively, while the dark gray arrow denotes the aglycoform (0 glycan). The dotted lines denote different lanes and the solid line represents a different gel. The numbers adjacent to the blots indicate the 150 kDa Kaleidoscope marker.
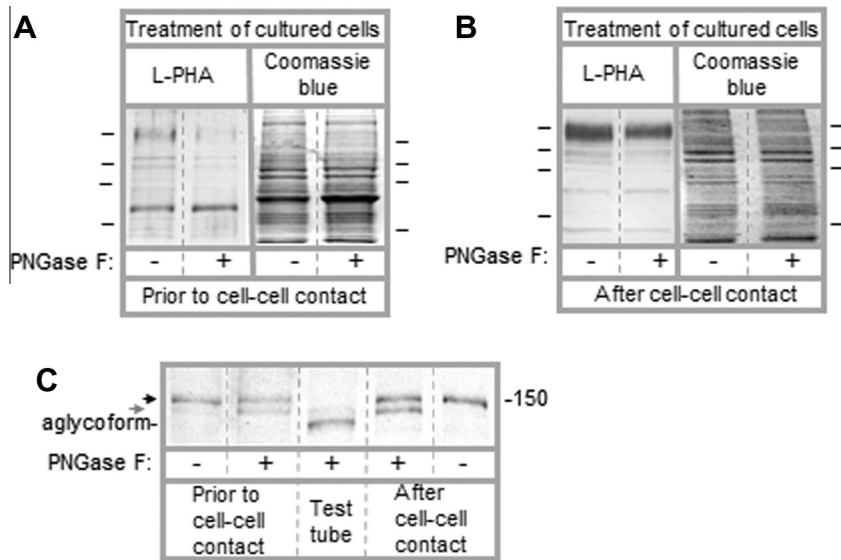
Fig. 2. Blots of glycoproteins from cultured cells treated with an endoglycosidase. Lectin blots of whole cell lysates (∼25 μg) from E-cadherin transfected Pro⁻5 CHO cells treated without (−) and with (+) PNGase F prior to (A) and after (B) formation of cell–cell contacts. Blots were probed with L-PHA (∼25 μg/mL). Gels loaded with similar amounts of electrophoresed proteins were also stained with Coomassie blue (A and B). Horizontal lines denote the Kaleidoscope markers (in kDa) from top to bottom (150, 100, 75, and 50). Western blots of E-cadherin from total membranes from E-cadherin transfected Pro⁻5 CHO cells treated without (−) and with (+) PNGase F prior to and after formation of cell–cell contacts, as indicated (C). Total membranes of E-cadherin transfected Pro⁻5 CHO cells were also digested with PNGase F (test tube) to denote the electrophoretic mobility of unglycosylated E-cadherin (aglycoform). Black and gray arrows point to the fully and partially glycosylated E-cadherin proteins, respectively. Similar findings were observed on at least three separate blots.

populations, respectively. A *p* value at 0.05 was used otherwise indicated.

3. Results and discussion

3.1. Cultured cells treated with PNGase F had reduced N-glycans at the cell surface

In several cases, N-glycosylation of transmembrane proteins have been shown to enhance their expression in the plasma membrane [18,19]. More recently, our studies revealed that distinct N-glycan structures contain information for the clustering of E-cadherin and Kv3.1, a voltage-gated K⁺ channel, to the cell–cell interface of epithelial-derived cells [12]. To directly demonstrate the impact of N-glycans at the cell surface in sequestering E-cadherin to the cell–cell border, they were reduced by treating live EGFP tagged E-cadherin transfected Pro⁻5 CHO cells with PNGase F (removes complex, hybrid and oligomannose N-glycans). The amount of PNGase F to treat live CHO cells was determined by employment of Kv3.1b transfected ProV cells. The different glycosylated and unglycosylated forms of Kv3.1b can easily be resolved on reducing SDS-gels. In contrast, separation of the glycosylated and unglycosylated forms of E-cadherin is much more difficult. Western blots of total membranes from cultured Kv3.1b transfected Pro⁻5 cells were treated with various amounts of PNGase F after formation of cell–cell contacts (Fig. 1). Since 10 U/mL of PNGase did not remove much of the N-glycans from Kv3.1b, and 75 and 100 U/mL resulted in protein degradation, 50 U/mL of PNGase F was employed to treat cultured Pro⁻5 cells.

Whole cell lysates from E-cadherin transfected Pro⁻5 cells that underwent treatment in the absence (−) and presence (+) of PNGase F were used for lectin blotting. PNGase F was added to cultured cells either before (Fig. 2A) or after (Fig. 2B) the establishment of cell–cell contacts. This allowed us to ascertain whether cell surface N-glycans controlled the recruitment and/or retention of E-cadherin at the cell–cell border. *Phaseolus vulgaris* Leucoagglutinin (L-PHA) was the lectin employed for blotting glycoproteins

since the far majority of N-glycans from Pro⁻5 cells are of complex type [20,21]. L-PHA interactions with glycoproteins from transfected Pro⁻5 cells treated with PNGase F prior to and after cell–cell contact were weaker than those from cells treated without PNGase F. In both cases, SDS-gels were stained with Coomassie blue to show that similar amounts of proteins were loaded.

Western blots of total membranes from E-cadherin transfected Pro⁻5 cells that underwent treatment in the absence of PNGase F before establishment of cell–cell contact, as well as after formation of cell–cell contact, revealed a major E-cadherin immunoband (Fig. 2C). In both cases, treatment with PNGase F generated a doublet of equal intensities, indicating that some of the N-glycans are removed from E-cadherin. We attribute the detection of the lower faint band in the samples treated without PNGase F to the lack of serum in the medium for 4 h. The electrophoretic migration of unglycosylated E-cadherin (aglycoform) was slightly faster than both immunobands, indicating that the lower band generated by PNGase F treatment is partially glycosylated E-cadherin. It may be that some of the N-glycans of E-cadherin are not accessible to the glycosidase when it is localized in the plasma membrane of a viable cell. For instance, the X-ray crystal structure of the ectodomains of E-cadherin suggested that cis interactions between E-cadherin proteins on the cell membrane form a crystal lattice [22]. Hence, the results indicate that some of the N-glycans were removed from the cell surface of live cells treated with PNGase F prior to and after formation of cell–cell contacts, and furthermore both procedures generated some partially glycosylated E-cadherin protein at the cell surface. As such, these protocols can be utilized to study how reduction in cell surface N-glycans alters various cellular properties and functions.

3.2. Reduction of cell surface N-glycans cause lower levels of E-cadherin at the cell–cell border

TIRF and DIC microscopic techniques were employed to determine the level of EGFP tagged E-cadherin protein at the cell–cell border of live cells as we previously described [12,17]. Images of
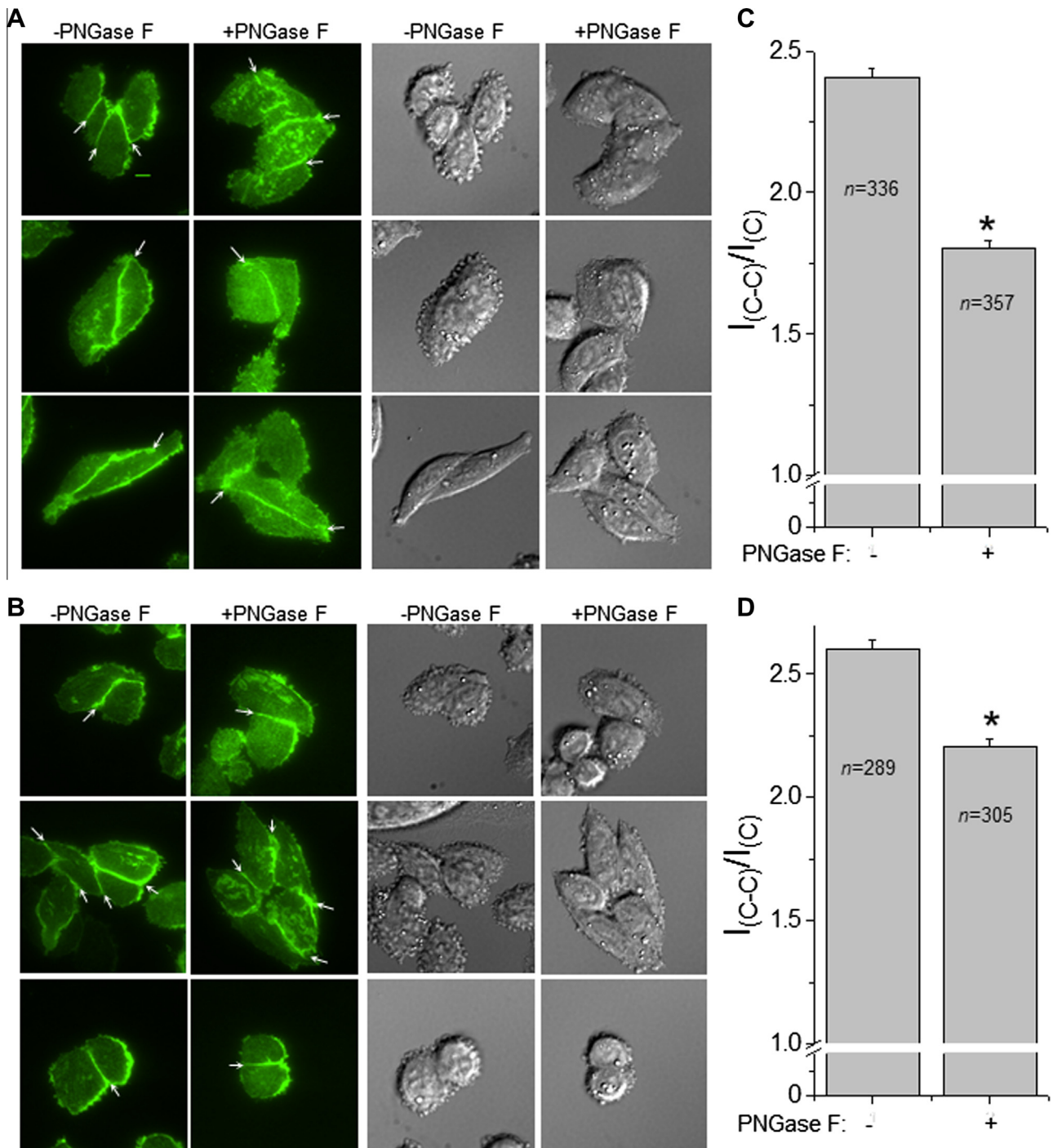
Fig. 3. Localization of E-cadherin at the cell–cell border in live cells treated with PNGase F. Representative TIRF (colored) and DIC (gray) microscopic images of EGFP tagged E-cadherin transfected Pro⁻5 cells treated without and with PNGase F prior to (A) and after (B) establishment of cell–cell contacts as indicated. Representative scale bar (5 µM) was identical for all images. White arrows identify cell–cell interface. Bar graphs represent the fluorescence intensity measurement at the cell–cell interface ($I_{cell–cell}$) divided by that away from the cell–cell interface (I_{cell}) of the cell membrane patch of E-cadherin expressed in Pro⁻5 cells treated before (C) and after (D) cell–cell contacts were established. At the 0.00001 level, the differences of the population means are significantly different by Student t-test (*). Data was collected from at least 3 experiments and n denotes the number of measurements.

cells in similar planes were also obtained in the wide-field mode (not shown) to verify that E-cadherin was in or near the plasma membrane in the TIRF mode. Representative TIRF (RGB color mode) and DIC (grayscale mode) images of E-cadherin transfected cells treated without (−) and with (+) PNGase F before (Fig. 3A) and after (Fig. 3B) formation of cell–cell contacts are shown. In all cases, the fluorescence signal of the adherent membrane patch was more concentrated at the cell–cell border than free of this

border. However, the fluorescence signal at the cell–cell border was weakened and that free of the cell–cell interface was strengthened in transfected Pro⁻5 cells treated with PNGase F relative to those cells treated without PNGase F. Both regions of interest were verified by the accompanied DIC images. To further quantify the levels of E-cadherin at and free of the cell–cell border, we determined the ratio of the fluorescence intensity signal at the cell–cell border ($I_{cell–cell}$) of the membrane patch to that free of this border
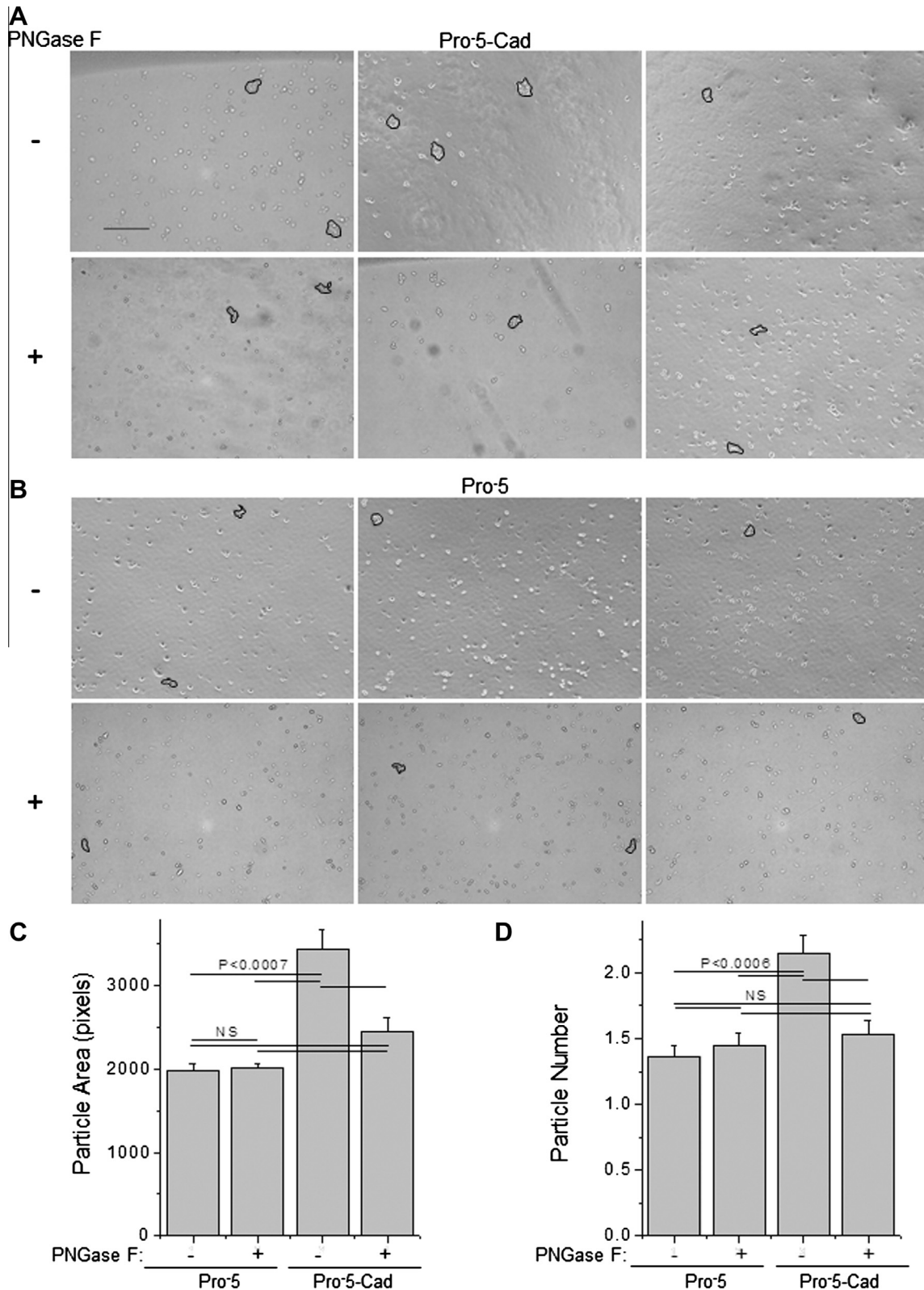
Fig. 4. Changes in levels of cell surface glycans alter E-cadherin mediated cell–cell adhesion. Typical microscopic images were acquired for E-cadherin transfected (A) and nontransfected (B) Pro⁻5 cells without (−, upper panel) and with (+, lower panel) PNGase F. Particles (>5 cells) of interest are encircled. Particle area (C) and particle number (D) of nontransfected and transfected CHO cells minus and plus PNGase F as indicated. Bar graphs represent data collected from 5 experiments and $n \geqslant 247$. One-way ANOVA with Bonferroni adjustment was used for statistical analysis. NS denotes means are not significantly different at a probability of $P < 0.1$.

(I_{cell}) [12]. When cells were treated with PNGase F, there was a 43% and 24% reduction in the amount of fluorescence signal at the cell–cell border for cells treated with PNGase prior to (Fig. 3C) and after (Fig. 3D) cell–cell contacts were established, respectively, relative

to those cells without PNGase F treatment. Hence, our results indicate that the amount of N-glycans at the cell surface has a role in regulating the clustering of E-cadherin to the cell–cell interface, along with the retention of E-cadherin at this interface.

Weak cis interactions of E-cadherin proteins in cell membranes have been proposed to give rise to a crystal lattice which supports E-cadherin clustering at the cell–cell border while the involvement of N-glycans in formation of this structure was not studied [22]. However, glycan to glycan interactions at the cell surface have been proposed to contribute to membrane organization [23]. Our past study revealed that N-glycan structures can alter the level of E-cadherin clustering at the cell–cell border [12]. Our current study directly demonstrates that N-glycan interactions at the cell surface participate in the clustering of E-cadherin to the cell–cell border.

3.3. Weaker cell–cell interactions are associated with lower levels of N-glycans at the cell–cell border

To ascertain whether reduced levels of cell surface N-glycans correlated with lower amounts of functional E-cadherin at the cell–cell interface, we measured the strength of E-cadherin mediated cell–cell interactions. Representative images of cell dissociation assays are shown for E-cadherin transfected (Fig. 4A) and nontransfected (Fig. 4B) Pro⁻5 cells treated without (−) and with (+) PNGase F. Size of particles were quite similar for nontransfected Pro⁻5 cells undergoing PNGase F treatment while the particle area of E-cadherin transfected Pro⁻5 cells treated without PNGase F were about 30% larger than those cells treated with PNGase F (Fig. 4C). Further particle area of E-cadherin transfected Pro⁻5 cells treated with PNGase F was close to the particle area of nontransfected Pro⁻5 cells treated with and without PNGase F. The particle number of E-cadherin transfected Pro⁻5 cells treated without PNGase F was about 2 while it was about 1.5 for all other cases (Fig. 4D). These results indicate that N-glycans at the cell surface influence E-cadherin mediated cell–cell adhesion while the effect on cell–cell interactions independent of E-cadherin was negligible.

Partially N-glycosylated and unglycosylated forms of the E-cadherin protein decrease, if not abolish, cell surface expression of the protein [11]. Our recent study revealed that distinct N-glycan structures dramatically altered the spatial arrangement of E-cadherin in the plasma membrane when cell–cell interactions were present while changes in lateral distribution of the E-cadherin glycoprotein were quite subtle in single cells, suggesting that information of N-glycans at the cell surface is decoded in the presence of cell–cell interactions. In this study, we have directly demonstrated that N-glycans at the cell surface play a role in the recruitment and retention of E-cadherin to the cell–cell border. Further increased levels of E-cadherin at this border led to enhanced cell–cell interactions. Taken together, these results indicate that the level and type of N-glycans outside the cell have a role in plasma membrane structure, and in the strength of E-cadherin mediated cell–cell interactions.

Acknowledgment

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