NEU1 Sialidase Associates with the MUC1 Cytoplasmic Domain and not the MUC1 Ectodomain

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ABSTRACT

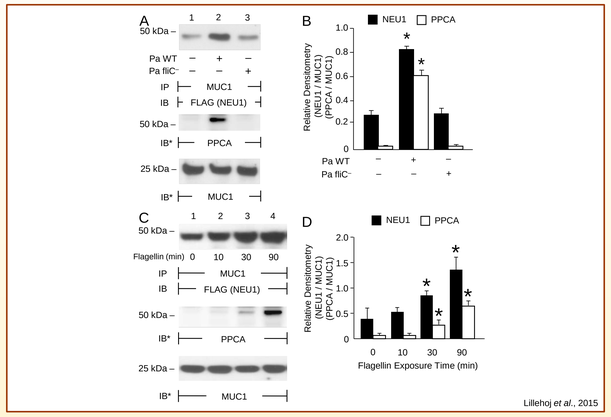
Background: The highly O-glycosylated MUC1-ectodomain (MUC1-ED) binds to *Pseudomonas aeruginosa* (Pa) flagellin, the major structural protein of the bacterial flagellar filament. In the respiratory tract, Pa is one of the most frequent and deadly causes of ventilator-associated pneumonia, and contributes to the pathogenesis of cystic fibrosis, bronchiectasis, and chronic obstructive pulmonary disease. We previously established that the MUC1-ED is an *in vivo* substrate for NEU1, the major sialidase expressed in human airways. NEU1 desialylates the MUC1-ED, exposing additional binding sites for Pa flagellin and increasing the affinity of the MUC1-ED-flagellin interaction. NEU1-mediated MUC1-ED desialylation also unmasks a Gly-Ser protease recognition site to allow its proteolytic release from the cell surface. These combined effects lead to the generation of a shed, hyperadhesive MUC1-ED decoy receptor that blocks flagellin-mediated Pa adhesion to the cell-associated MUC1-ED and protects against lethal Pa lung infection. While NEU1 was initially described as an intracellular enzyme localized to the lysosomes, sialic acid residues on the MUC1-ED are located in the extracellular compartment. The pathway through which cytoplasmic NEU1 might be translocated to the extracellular sialic acid residues of the MUC1-ED substrate is incompletely understood. Therefore, the current studies were undertaken to elucidate the mechanism through which NEU1 is recruited to MUC1 leading to Pa flagellin-induced generation of the shed, hyperadhesive MUC1-ED decoy receptor.

Results: We asked whether preformed pools of NEU1 and/or its transport/chaperone protein, protective protein/cathepsin A (PPCA), might be mobilized in response to the Pa stimulus. Incubation of A549 human airway epithelial cell cultures with flagellin-expressing Pa, but not with the Pa fliC^{-} flagellin-deficient isogenic mutant, increased MUC1-NEU1 and MUC1-PPCA co-immunoprecipitation compared with the simultaneous medium controls (Figure 1). Increased MUC1-NEU1 and MUC1-PPCA co-immunoprecipitation also was seen in lung tissues using an *in vivo* mouse model of Pa pneumonia, compared with unchallenged mice (Figure 2). For intracellular NEU1 to gain access to the surface-expressed, sialylated MUC1-ED substrate, we asked whether NEU1 might associate with MUC1-ED and/or MUC1-CD using *in vitro* protein binding assays with GST-NEU1 immobilized on glutathione-agarose. GST-NEU1 associated with the MUC1-CD, but not with the MUC1-ED, in lysates of HEK293T cells transfected for expression of either MUC1-ED or MUC1-CD (Figure 3). In the reciprocal approach, NEU1 binding to 6X-His-MUC1-ED immobilized on Ni-NTA-agarose was not detected. To address the possibility that a conformational restraint was hindering NEU1 association with the full-length MUC1-ED protein, reciprocal GST- and Ni-NTA-pull down assays were performed with deletion mutants encompassing the 375-amino acid (aa) MUC1-ED. Again, no protein association was detected (Figure 4). To further support a NEU1-MUC1-CD interaction, and to begin to define the MUC1-CD structural requirements for its association with NEU1, we constructed GST fusion proteins encompassing the entire 72-aa intracellular region of MUC1-CD (aa 1-72), its 36-aa membrane-proximal NH_2-terminal half (aa 1-36), and its 36-aa COOH-terminal half (aa 37-72). NEU1 associated with GST-MUC1-CD (aa 1-72) and with GST-MUC1-CD (aa 1-36), but not with GST-MUC1-CD (aa 37-72) (Figure 5), mapping the NEU1 binding site to the NH_2-terminal juxtamembrane half of the MUC1-CD. Finally, purified NEU1 that was proteolytically cleaved from its GST epitope tag by Factor X and purified on a GST trap column, associated with GST-MUC1-CD (aa 1-72) immobilized on glutathione-agarose (Figure 6). In the reciprocal approach, purified GST-MUC1-CD (aa 1-72) that was purified by binding to and elution from glutathione-agarose associated with 6X-His-NEU1 immobilized on Ni-NTA-agarose.

Conclusions: Combined with our prior studies, these results indicate that binding of Pa flagellin to the cell surface MUC1 receptor rapidly stimulates the recruitment of a preformed, intracellular pool of NEU1 to the juxtamembranous portion of the MUC1-CD, which in turn, leads to NEU1-mediated MUC1-ED desialylation.

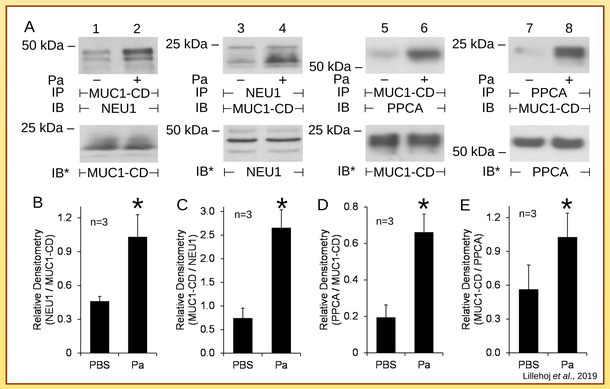
RESULTS

Figure 1. Pa and its flagellin promote NEU1 and PPCA association with MUC1 *in vitro*.



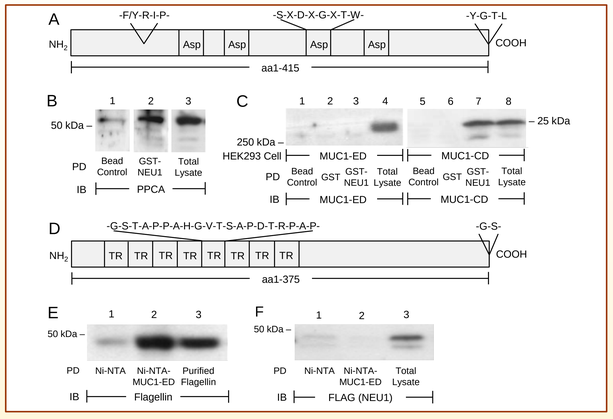
(A) A549 cells were infected with Ad-NEU1-FLAG and incubated with wild-type Pa (lane 1), the flagellin-deficient Pa fliC^{-} isogenic mutant (lane 2), or medium alone (lane 3) and lysed. The lysates were immunoprecipitated with anti-MUC1 Ab and the immunoprecipitates processed for FLAG (NEU1) immunoblotting (upper panel). The blots were stripped and reprobed for PPCA (middle panel) or MUC1 (lower panel). (B) Densitometric analysis. (C) A549 cells were infected with Ad-NEU1-FLAG and incubated with medium alone (lane 10 or for increasing times with Pa-derived flagellin (lanes 2-4) and lysed. The lysates were immunoprecipitated with anti-MUC1 Ab and the immunoprecipitates processed for FLAG (NEU1) immunoblotting (upper panel). The blots were stripped and reprobed for PPCA (middle panel) or MUC1 (lower panel). (D) Densitometric analysis.

Figure 2. Pa drives NEU1 and PPCA association with MUC1 *in vivo*.



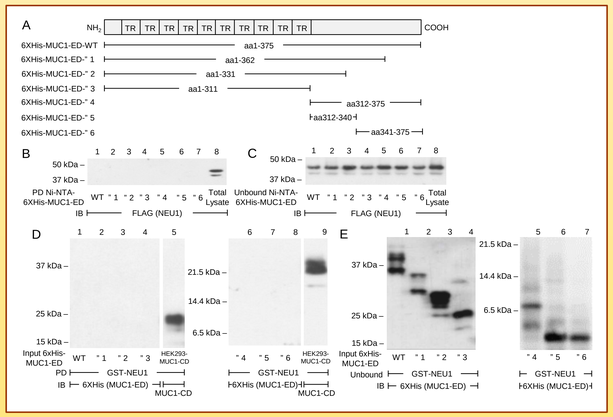
BALB/c mice were administered i.n. with 1.0 x 10^5 CFUs/mouse of Pa or PBS. At 24 h post-administration, lung tissues were collected and homogenized. (A) Lung homogenates were immunoprecipitated with anti-MUC1-CD Ab (lanes 1, 2, 5, 6), anti-NEU1 Ab (lanes 3, 4), or anti-PPCA Ab (lanes 7, 8). The MUC1-CD immunoprecipitates were processed for NEU1 (lanes 1, 2) or PPCA (lanes 5, 6) immunoblotting, and the NEU1 (lanes 3, 4) and PPCA (lanes 7, 8) immunoprecipitates processed for MUC1-CD immunoblotting (upper panels). To control for equal protein and loading, the blots were stripped and reprobed for the immunoprecipitating Ab (lower panels). (B-E) Densitometric analysis.

Figure 3. NEU1 associates with the MUC1-CD and not with the MUC1-ED.



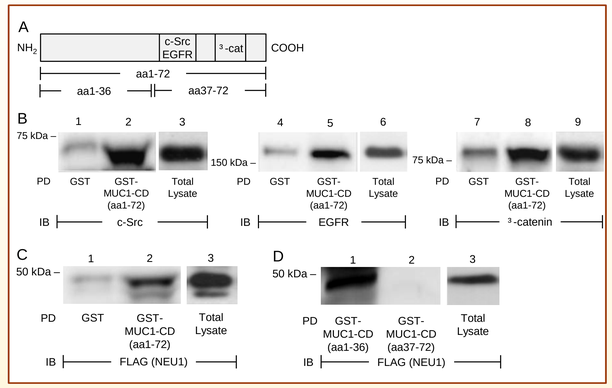
(A) Schematic of NEU1. (B) Validation of the GST-NEU1 construct for binding to PPCA. (C) Lysates of HEK293T cells transfected for MUC1-ED (lanes 1-4) or MUC1-CD (lanes 5-8) expression were incubated with beads alone (lanes 1, 5), or with GST (lanes 2, 6) or GST-NEU1 (lanes 3, 7) immobilized on the beads, or directly loaded on the gel (lanes 4, 8). Proteins bound to the beads and total lysates were processed for MUC1-ED or MUC1-CD immunoblotting. (D) Schematic of the MUC1-ED. (E) Validation of the 6X-His-MUC1-ED construct for binding to Pa flagellin. (F) Lysates of A549 cells infected with Ad-NEU1-FLAG were incubated with Ni-NTA-agarose beads alone (lane 1) or 6X-His-MUC1-ED immobilized on the beads (lane 2), or directly loaded on the gel (lane 3). Proteins bound to the beads and total lysates were processed for FLAG (NEU1) immunoblotting.

Figure 4. NEU1 fails to associate with MUC1-ED deletion mutants.



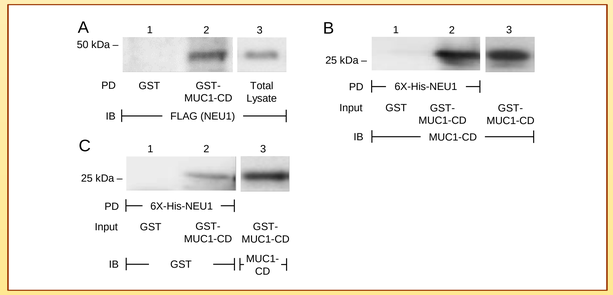
(A) Schematic of MUC1-ED and deletion mutants. (B, C) Lysates of A549 cells infected with Ad-NEU1-FLAG were incubated with 6XHis-MUC1-ED or deletion mutants, each immobilized on Ni-NTA-agarose beads (lanes 1-7), or directly loaded on the gel (lane 8). (B) Proteins bound to the beads, (C) proteins unbound to the beads, and total lysates were processed for FLAG (NEU1) immunoblotting. (D, E) 6XHis-MUC1-ED and deletion mutants were adsorbed to Ni-NTA-agarose beads and eluted with imidazole. The purified proteins (lanes 1-4, 6-8), or lysates of HEK-293T transfected for MUC1-CD expression (lanes 5, 9), were incubated with GST-NEU1 immobilized on glutathione-agarose beads. (D) Proteins bound to the beads, (E) proteins unbound to the beads, and HEK-293T cell lysates were processed for 6XHis (MUC1-ED) or MUC1-CD immunoblotting.

Figure 5. NEU1 associates with the juxtamembranous region of the MUC1-CD.



(A) Schematic of the MUC1-CD. (B) Validation of the GST-MUC1-CD (aa1-72) construct for binding to c-Src, EGFR, and ³-catenin. (C) Lysates of A549 cells infected with Ad-NEU1-FLAG were incubated with GST (lane 1) or GST-MUC1-CD (aa1-72) (lane 2) each immobilized on glutathione-agarose beads, or directly loaded on the gel (lane 3). Proteins bound to the beads and total lysates were processed for FLAG (NEU1) immunoblotting. (D) Lysates of A549 cells infected with Ad-NEU1-FLAG were incubated with GST-MUC1-CD (aa1-36) (lane 1) or GST-MUC1-CD (aa37-72) (lane 2) each immobilized on glutathione-agarose beads, or directly loaded on the gel (lane 3). Proteins bound to the beads and total lysates were processed for FLAG (NEU1) immunoblotting.

Figure 6. NEU1 associates directly with the MUC1-CD.



(A) Lysates of A549 cells infected with Ad-NEU1-FLAG were incubated with GST (lane 1) or GST-MUC1-CD (aa1-72) (lane 2), each immobilized on glutathione-agarose beads. The beads were incubated with Factor X to proteolytically release the bound proteins and the released proteins purified on a GST trap column. The purified proteins and total lysates (lane 3) were processed for FLAG (NEU1) immunoblotting. (B, C) GST (lane 1) and GST-MUC1-CD (lane 2), each coupled to glutathione-agarose beads, were eluted with free glutathione and incubated with 6X-His-NEU1 coupled to Ni-NTA-agarose beads. The 6X-His-NEU1-binding proteins, and purified GST-MUC1-CD (lane 3), were processed for (B) MUC1-CD or (C) GST immunoblotting.

Acknowledgments

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