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Modulation of host cell endocytosis by the type-III cytotoxin, *Pseudomonas* ExoS

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Abstract

Pseudomonas aeruginosa ExoS is a bi-functional type III cytotoxin that possesses Rho GTPase activating protein (RhoGAP) and ADP-ribosyltransferase (ADPr) activities. In the current study, the RhoGAP- and ADPr- activities of ExoS were tested for the ability to disrupt mammalian epithelial cell physiology. RhoGAP, but not ADPr, inhibited internalization/phagocytosis of bacteria, while ADPr, but not RhoGAP, inhibited vesicle trafficking, both general fluid phase uptake and epidermal growth factor (EGF)-activated EGF receptor (EGFR) degradation. In ADPr-intoxicated cells, upon EGF activation, EGFR co-localized with clathrin coated vesicles (CCV) which did not mature into Rab5 positive early endosomes. Constitutively active Rab5 recruited EGFR from CCV to early endosomes. Consistent with the inhibition of Rab5 function by ADPr, several Rab proteins including Rab5 and 9, but not Rab4, were ADP ribosylated by ExoS. Thus, the two enzymatic activities of ExoS have different effects on epithelial cells with RhoGAP inhibiting bacterial internalization and ADPr interfering with CCV maturation. The ability ADP-ribosylation to inhibit mammalian vesicle trafficking provides a new mechanism for bacterial toxin-mediated virulence.

Keywords

ExoS; Rab 5; endocytosis; epidermal growth factor receptor; fluid phase uptake

Introduction

Pseudomonas aeruginosa is a Gram negative, opportunistic pathogen that infects compromised patients, including those with severe burn wounds or cystic fibrosis, causing both acute and chronic infections(1). *P. aeruginosa* type III secretion system (T3SS) delivers four exotoxins into host cells(2): ExoS, ExoT, ExoU(3) and ExoY (4), which create an environment that favors bacterial survival and dissemination. ExoS and ExoT are bi-functional toxins comprising a Rho GTPase Activating Protein (RhoGAP) domain and an ADP-ribosyltransferase (ADPr) domain(5). ExoS- and ExoT- RhoGAPs inactivate small Rho GTPases, including RhoA, Rac1, and Cdc42, disrupting the host cell cytoskeleton(5). ExoS ADP-ribosylates numerous substrates, including vimentin(6), Ras small GTPases (7), ERM(8), and cyclophilin A(9), eliciting a cytotoxic phenotype in cultured cells, while ExoT ADP-ribosylates a more restricted group of substrates, including CrkI and CrkII(10), uncoupling intergrin mediated signaling (11).

Rab proteins are a family of small G proteins (Rab GTPases) that regulate vesicular budding and fusion reactions by oscillating between active, GTP and inactive, GDP, bound forms(12).

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Rab GTPases play an essential role in host cell vesicle trafficking including endocytosis, receptor recycling, vesicle maturation and trafficking to Golgi or lysosomes(13). The coincidence of ExoS localization to Rab positive vesicles and identification that Rab5, Rab7, Rab8 and Rab11 are ADP-ribosylated by ExoS *in vitro* and *in vivo*(15) promoted the hypothesis that ExoS ADP-ribosylates Rab GTPases to inhibit host cell vesicle trafficking pathways. *In vitro*, ExoS uncoupled Rab5-early endosome antigen 1 interaction and inhibited fluid phase uptake (14). While an informative study, Vidal et al (14) conducted experiments with full-length ExoS and did not resolve the molecular basis for the observed phenotypes as being due to RhoGAP or ADP-r activities. Furthermore, the ability of ExoS ADPr to disrupt receptor-mediated trafficking in cultured cells has not been described.

Human epidermal growth factor receptor (EGFR) is a 180-kDa cell surface receptor tyrosine kinase that functions in the growth and development of differentiated cells. EGFR exists as a monomeric, single membrane spanning protein in the resting state(16). Upon binding of EGF to the extracellular domain, the EGFR dimerizes, which initiates autophosphorylation and signaling events that lead to cell proliferation, migration, and differentiation(17). EGF stimulation also triggers the internalization of the EGF-EGFR complex via the canonical clathrin-mediated endocytic pathway(18), through early and late endosomes for degradation in lysosomes(19). Endocytic trafficking of the EGFR is necessary for the proper temporal and spatial regulation of EGF signaling. Several G proteins have been implicated in EGFR endocytic trafficking. While Dynamin coordinates EGFR endocytosis (20), Rab5 mediates entry of the EGFR into early endosomes(21,22). Rab7 is required for the degradation of the EGF·EGFR complex by the lysosomes(23) and Rab11 facilitates EGFR recycling to the plasma membrane(24).

In this study, the ability of ExoS RhoGAP- and ADPr-activities to modulate bacterial internalization and vesicle trafficking in host cells were investigated.

Results

ExoS RhoGAP inhibits the internalization of *P. aeruginosa* by HeLa cells

To dissect the role of ExoS RhoGAP and ADPr activities on host cell function, *P. aeruginosa* expressing ExoS RhoGAP (E381D, mutation within the active site for ADPr domain, termed G^+) or ADPr (R146K, mutation in the active site for RhoGAP domain, termed A^+) were constructed. Internalization of bacteria by HeLa cells was used to determine the role of ExoS in the anti-internalization activity of *P. aeruginosa*. Wild type ExoS (G^+A^+) reduced bacteria internalized by ~2 logs compared with *P. aeruginosa* expressing a vector control (pUCP), while catalytically null ExoS (R146K, E379/381D, termed G^-A^-) did not inhibit *P. aeruginosa* internalization (Figure 1B). This linked the anti-internalization function to ExoS enzymatic activities. ExoS G^+ inhibited bacteria internalization to a similar extent as ExoS G^+A^+, while ExoS A^+ showed a similar phenotype as ExoS G^-A^-, indicating that RhoGAP activity alone contributed to the ExoS anti-internalization activity. In addition, a truncated form of ExoS, lacking the C-terminal ADPr domain (ExoS 1–234), inhibited *P. aeruginosa* internalization to a similar level as ExoS G^+ (Figure 1B), indicating that the ADPr domain was dispensable for anti-internalization function. Nocodazole (noco), a microtubule destabilizing reagent and cytochalasin D (cytoD), an actin inhibitor were used as negative or positive controls, respectively, where cytoD showed ~10 fold reduction of *P. aeruginosa* (pUCP) internalization by HeLa cells, consistent with the role of actin in phagocytosis. Immunoblot showed the amount of ExoS derivatives delivered into HeLa cells where GAPDH showed comparable loading (Figure 1A). Thus, ExoS anti-internalization activity is dependent on ExoS RhoGAP, but not ADPr activity.

P. aeruginosa ExoS ADPr activity inhibits fluid phase uptake of HeLa cells

Horse radish peroxidase (HPR) uptake was used to measure early endosome function in HeLa cells intoxicated with ExoS derivatives. HeLa cells intoxicated with *P. aeruginosa* expressing pUCP or ExoS G^-A^- took up HRP at levels similar to uninfected cells (-inf), while HeLa cells intoxicated with ExoS G^+A^+ showed ~30% reduction in HRP uptake (Figure 2), suggesting ExoS inhibition of HRP uptake was activity dependent. ExoS A^+ inhibited HRP uptake by 40% (Figure 2), but neither ExoS G^+ nor ExoS(1–234) inhibited HRP uptake significantly, although ExoS G^+ and ExoS(1–234) intoxicated cells showed a rounding phenotype (data not shown). This indicated that ExoS ADPr activity was responsible for fluid phase uptake inhibition. Noco and cytoD were used as positive and negative controls, respectively, to implicate a role for microtubule, but not actin, in the fluid phase uptake in HeLa cells.

P. aeruginosa ExoS ADPr activity inhibits EGF-activated EGFR degradation in HeLa cells

The impact of ExoS ADPr activity on host vesicle trafficking was further tested by measuring EGF-activated EGF receptor (EGFR) degradation, a pathway involving clathrin mediated uptake of activated EGFR dimer, maturation to early and late endosomes and final degradation of the EGFR in lysosomes. Initial experiments observed that the steady state level of EGFR was reduced with the addition of EGF (Figure 3, immunoblot). EGF-activated EGFR degradation was inhibited by lysosome inhibitors, bafilomycin A1 and chloroquine, but not the proteasome inhibitor, clasto-lactacyctin β-lactone (data not shown), supporting a lysosomal pathway for EGFR degradation(25). HeLa cells intoxicated with ExoS G^+A^+ or ExoS A^+ inhibited EGF-activated EGFR degradation by ~35%. This inhibition was not observed in HeLa cells intoxicated with *P. aeruginosa* expressing pUCP vector control, ExoS G^-A^- or ExoS G^+, indicating that the inhibition of EGFR degradation was dependent on the expression of ADPr activity (Figure 3). Furthermore, EGFR was phosphorylated at Tyrosine 1068 in HeLa cells intoxicated with ExoS A^+ upon EGF activation (Supplemental Figure 1). This indicated that the inhibition of receptor degradation was not due to failure of EGF to bind to the receptor and stimulate EGFR phosphorylation to initiate the degradation signal.

P. aeruginosa ExoS ADPr inhibits EGF-activated EGFR trafficking to lysosomes

To determine how ADP-ribosylation inhibited EGFR degradation, EGF-activated EGFR localization was measured in HeLa cells intoxicated with *P. aeruginosa* expressing ExoS derivatives. Wheat germ agglutinin (WGA) was used as a plasma membrane marker (Figure 4) and Lamp 1 was used as lysosome marker (Figure 5). In unstimulated cells, EGFR localized on the plasma membrane with a small intracellular pool, whereas upon EGF-activation, plasma membrane localized EGFR was not detected (Figure 4). In HeLa cells intoxicated with ExoS G^+ or ExoS A^+, EGF activated the internalization of the EGFR, indicating movement of the EGFR off the plasma membrane and into the endocytic pathway.

In cells intoxicated with ExoS G^-A^- or ExoS G^+, EGF stimulated the EGFR to traffic to lysosomes (ICQs=~0.2, indicating partial co-localization), while in cells intoxicated with ExoS A^+, upon EGF stimulation EGFR was excluded from lysosomes (ICQ=~-0.1, indicating segmentation) (Figure 5). In these experiments rounded cells (stars) were scored for EGFR localization, since ExoS intoxication induces cell rounding through either RhoGAP- or ADPr-activities(26,27). Taken together, ExoS ADPr blocks EGF-activated EGFR trafficking to lysosomes, rather than blocking EGFR internalization.

P. aeruginosa ExoS ADPr activity inhibits EGF-activated WGA uptake of HeLa cells

WGA, which labels plasma membrane glycol-proteins and glycol-lipids, were also internalized upon EGF activation (Figure 4). In contrast to EGFR whose internalization was independent of ExoS ADPr activity, WGA internalization was blocked by ADPr activity, with 15% of ExoS

A^+ intoxicated HeLa cell having intracellular WGA, compared to 76% and 93% of cells intoxicated with ExoS G^-A^- or ExoS G^+. Similar to fluid phase uptake which is enhanced with EGF activation, WGA uptake was also unregulated by EGF, possibly through the activation of Ras protein which induced membrane ruffles and enhanced fluid phase uptake(28).

ExoS ADPr inhibits EGFR trafficking by inhibiting Rab5 function. To determine how ADPr inhibited EGFR traffics to the lysosome, the intracellular localization of EGFR after EGF-activation in ExoS A^+ intoxicated cells was determined. Sixty seven percent of the intoxicated cells accumulated EGFR in the cell periphery adjacent to the plasma membrane (Figure 6A), suggesting that ADPr inhibited an early step in EGFR trafficking. Co-localization experiments observed that EGFR co-localized with clathrin heavy chain (ICQ=0.31±0.04), but not with Rab5 (ICQ=0.021±0.02), indicating EGFR trafficking from clathrin coated vesicles (CCV) to Rab5 positive vesicles was blocked. In contrast, in ExoS G-A- or ExoS G+ intoxicated cells, EGFR colocalized with clathrin to a lesser extent (Supplemental Figure 2), This may be due to flat clathrin coats that are proposed to be present on endosome microdomains to mediate protein sorting to degradative pathways(29).

To test if the inhibition of Rab5 function was the mechanism of ExoS ADPr action on vesicle trafficking, a GFP-fused constitutively active Rab5 protein (GFP-Rab5-Q79L, termed DA-Rab5) was transiently expressed in HeLa cells and the ability of DA-Rab5 to recruit EGFR was measured. Expression of DA-Rab5 leads to enlarged Rab5 positive early endosomes due to continuous vesicle fusion(30). In ExoS A^+ intoxicated cells, 89% of the enlarged early endosomes recruited EGFR upon EGF activation (Figure 6B) and the co-localization efficiency of Rab5 and EGFR increased to ICQ=0.13±0.01. In an independent experiment, Rhodamine-conjugated EGF was used to monitor the localization of EGFR initiated from the plasma membrane, rather than from an intracellular pool. Eighty five % of DA-Rab5 positive vesicles recruited Rhodamine-conjugated EGF in cell intoxicated with ExoS A^+ (Figure 6B). Together, these data suggest that ExoS ADPr activity traps EGFR in CCVs by inhibiting Rab5 activity. Furthermore, 68% of DA-Rab5 vesicles positive for EGFR also accumulated detectable amounts of early endosome antigen 1 (EEA1), a Rab5 effector protein in ExoS A^+ intoxicated cells (Figure 7), which suggested a functional DA-Rab5 activity.

ExoS ADP-ribosylates Rab GTPase in *P. aeruginosa* intoxicated HeLa cells

The ability of ExoS to ADP-ribosylate Rab GTPase in HeLa cells was confirmed by immunoblotting endogenous Rab GTPases in cells intoxicated with ExoS. Consistent with previous reports (15), Rab5 and Rab9, but not Rab4 were shifted to a slower migrating band in cells intoxicated with ExoS G^+A^+ or ExoS A^+, but not with vector control, ExoS G^-A^- or ExoS G^+ (Supplemental Figure 3), indicating ExoS ADP-ribosylates specific Rabs in cultured HeLa cells.

Discussion

ExoS RhoGAP

Both ExoS and ExoT have conserved RhoGAP domains which act on the three major classes of Rho GTPases, among which, Rac 1 and Cdc42 are regulators of actin dynamics linked to phagocytosis. Thus, RhoGAPs act as potent anti-internalization reagents(31). Bacterial encoded RhoGAPs mimic mammalian RhoGAPs to facilitate bacterial survival in the host environment. Similar to ExoS, the RhoGAP activity of YopE is required for the extracellular pathogen, *Y. pseudotuberculosis* to elicit anti-phagocytic function and virulence(32), while in the intracellular pathogen *Salmonella* type III effector, SopE, a Rho GTP exchange factor, activates RhoA and Cdc42 to stimulate local actin polymerization and promote the uptake of

the bacteria into the cells and SptP, a RhoGAP, reverses SopE activation to maintain *Salmonella* in a replication competent vesicle (33).

ExoS ADPr

Although the anti-internalization activity for ExoS GAP is well documented (34,35), whether or not ExoS ADPr plays any role in bacterial internalization is not known. ExoT ADPr domain has an anti-internalization activity(31), via the ADP-ribosylation of Crk family proteins(10). Crk I and Crk II are adaptor proteins required for recruiting $_{p}130^{CAS}$ and Paxillin to activated focal adhesion sites(11), which lead to Rac 1 activation and thus phagocytosis. In contrast, ExoS ADP-ribosylates multiple substrates, including Ras and Ezrin/Radixin/Moesin (ERM) family proteins (8), which are regulators of actin network. However, ExoS ADP-r activity did not inhibit *P. aeruginosa* internalization, suggesting that ERM-regulated actin organization plays a limited role in regulating phagocytosis. Since most clinical isolates contain either ExoU or ExoS(36), in addition to ExoT, ExoS ADPr domain may play a distinct role, other than anti-internalization, to elicit virulence. In this study, ExoS A+ inhibited receptor-mediated endocytosis and general fluid phase uptake, which presents a new mechanism for ExoS virulence.

Role of Rab5 proteins in ExoS inhibition of host vesicle trafficking

In addition to the well characterized role of Rab5 in targeting plasma-membrane-derived vesicles to endosomes and in homotypic fusion between endosomes, Rab5 contributes to protein sorting, regulation of motility of early endosomes, signal transduction, receptor tyrosine kinase induced actin remodeling and clathrin mediated endocytosis(13). Rab proteins are ADP-ribosylated by ExoS *in vitro* and in *vivo*(15). Earlier study observed that ExoS diminished the interaction of Rab5 with EEA1 and reduced endosome-endosome fusions (14). The observation that ExoS ADP-ribosylated Rab5, together with the ability of constitutively active Rab5 protein to reverse ExoS inhibition of EGF-activated EGFR trafficking out of CCVs, suggests that ExoS ADPr inhibits Rab5 activity in cells.

ExoS inhibits CCV-EE fusion

In this study, in cells intoxicated with ExoS A^+, EGFR was trapped within CCVs. Galperin and Sorkin showed that in Porcine aortic endothelial cells overexpressing dominant negative Rab5(S34N), EGFR was retained in clathrin-coated pits(37), similar to the phenotype in ExoS A^+ intoxicated cells, which supports the determination that ExoS A^+ inactivates Rab5. The exact role of Rab5 in clathrin-mediated endocytosis is still debated. Over-expression of constitutively active Rab5-Q79L or dominant-negative Rab5-S34N (21) and Rab5-regulating proteins, such as Rab-GDI(38) and Rab5 GTPase-activating protein(39), reduced the rate and total amount of EGFR and Transferrin receptor (TfnR) internalization, suggesting a role of Rab5 activity in clathrin-dependent endocytosis. In contrast, over-expression of Rab5Q79L did not alter trafficking of the TfnR and dominant negative Rab5 did not inhibit EGFR endocytosis, but that Rab5 determined if internalized EGF/EGFR and Rab5 co-localize(40), implicating a role for Rab5 subsequent to the movement of EGFR from the plasma membrane. Rubini *et al* showed that Rab5 and EEA1 are required for CCV and EE fusion *in vitro*(41). The observation that ExoS A^+ inhibited the fusion of EGFR-containing vesicles to Rab5 positive early endosomes, rather than receptor endocytosis, is consistent with the role of Rab5 in CCV and EE fusion. However, a role for Rab5 in clathrin mediated endocytosis can not be completely ruled out since ExoS ADP-r may only disrupt a subgroup of Rab5 involved activities.

The current study is the first to observe that DA-Rab5 rescues the ExoS ADPr inhibition of EGFR trafficking in cultured cells. The observation that DA-Rab5 recruited EEA1, rescuing EGFR from CCVs to early endosomes (Figure 7) is consistent with the observation that ExoS

inhibited Rab5 interaction with EEA1 *in vitro*(14), although the molecular mechanism of this inhibition needs further characterization.

ExoS inhibits fluid phase endocytosis

Rab5 is also involved in the regulation of EGF induced fluid-phase endocytosis(21,42). Role of Rab5 in regulating endocytosis is supported by a presence of Rab5 exchange factors on the plasma membrane of *Caenorhabditis elegans* (43). While an earlier study implicated a role for ExoS in mediating an inhibition of HRP marked fluid-phase endocytosis(14), the current study showed that the ADP-r activity of ExoS was responsible for this inhibition.

ExoS A^+ also inhibited EGF-activated internalization of WGA, which labels glycosylated cell surface proteins and lipids. While expression of DA-Rab5 stimulated HRP uptake(14), DA-Rab5 did not restore EGF-activated internalization of WGA in ExoS A^+ intoxicated cells (data not shown), indicating that ExoS may inhibit another substrate for ADPr, such as Ras(28) to impair EGF-activated WGA uptake. EGF activated Ras induced formation of macropinosomes and increased the rate of fluid phase uptake (29). Our data indirectly imply a role for both Ras and Rab5 for EGF induced fluid phase uptake.

Summary

P. aeruginosa translocates ExoS through the type III secretion apparatus where ExoS transiently associates with plasma membrane. ExoS GAP inactivates RhoGTPase to impair local actin reorganization to inhibit bacterial internalization, while ExoS ADPr inhibits Rab5 function. Upon EGF activation, EGFR was internalized to CCVs, which did not mature to Rab5 positive early endosomes. ADPr-also inhibited fluid phase uptake and EGF-activated endocytosis of WGA labeled proteins and lipids at the plasma membrane. Taken together, ExoS inhibits CCV maturation to an early endosome in receptor mediated endocytosis and inhibits endocytosis at the plasma membrane during fluid phase uptake. Wiley and Cunningham compared the EGF dose dependency and time course of EGF-activated fluid phase endocytosis and endocytosis of the EGFR and found these two processes were not directly coupled(44). Thus, ExoS may inhibit these two endocytic processes by different mechanisms.

Maturation of the phagolysosome is orchestrated by Rab GTPases where Rab5 controls the early phases of phagosome maturation and Rab7 regulates fusion events between the late endosome and lysosome(45). Recently a *Listeria* GAPDH protein was reported to ADP ribosylate Rab5 and inhibit Rab5a-mediated phagosome–endosome fusion(46). Thus, Rab5 may be a common target for both Gram negative or Gram positive bacteria to inhibit phagosomal functions. For intracellular pathogens, this modification favors bacterial survival, while for extracellular pathogens, this modification may reduce antigen presentation of bacterial proteins, or maintain an intracellular supply of toxin at specific intracellular locations for regulated activities.

Materials and Methods

Materials

P. aeruginosa strain PA103 (Δ*exo*U, *exo*T::Tc) with the indicated ExoS derivative was maintained and cultured as described previously (5). Rabbit antibody against clathrin heavy chain and phospho-EGFR (P-Tyr1068) were from Cell Signaling Technology, Inc. Sheep polyclonal antibody against EGFR was from Upstate Cell Signaling Solutions. Mouse antibody against Rab9 was from Calbiochem. Mouse antibody against Rab4 and Rab5 were from BD Transduction Laboratories. Mouse antibody against LAMP 1(CD107a) was from BD

biosciences. EGF and Rhodamine conjugated EGF was from Invitrogen and chemicals were from Sigma.

Cell culture

HeLa cells (CCL-2) were from the ATCC. HeLa cells were cultured in Minimum Essential Medium (Invitrogen) supplemented with 10% fetal calf serum, non-essential amino acids, sodium pyruvate, sodium bicarbonate and penicillin/streptomycin and maintained humidified at 37°C in 10% CO_2 (v:v).

Transfection and Type III delivery of ExoS into HeLa cells

HeLa cells were seeded in 6 or 12 well dishes with 1.5–3×10^5 cells the day before use. Cells (~70% confluent) were transfected with the Lipofectamine/Plus-transfer system (Invitrogen) as described by the manufacturer. After 18–24 h, cells were intoxicated with the indicated strain of *P. aeruginosa* PA103 (Δ*exo*U, *exo*T::Tc). Intoxication experiments were performed at a multiplicity of 8:1 (bacteria:HeLa cells). Dishes were centrifuged at 400 ×g for 10 min at RT to synchronize the infection and incubated at 37°C for indicated time of intoxication.

HeLa cell *P. aeruginosa* internalization assay

This assay was modified from(31). HeLa cells, in 6 well plates, were co-cultured with *P. aeruginosa* expressing ExoS derived constructs at 37°C for 3 h. Cells were then washed and cultured in medium containing 300 μg/ml Gentamycin for 1 h. To ensure that invasion assays were not affected by loss of rounded, non-adherent cells, the supernatant of each well was collected in an Eppendorf tube. Adherent cells were trypsinized and combined with cells recovered from the supernatant. To release the internalized bacteria, cells were washed with PBS and suspended in 0.5 ml of PBS with 0.1% Triton X-100 (Sigma). After 15 min of incubation at RT, serial dilutions of the lysates were performed and 100 μl of each dilution were plated on LB agar and incubated at 37°C for 18 h to quantify the CFU of internalized bacteria. The invasion assays were normalized to the number of cells in each well, as determined by measuring the total amount of lactate dehydrogenase (using the CytoTox 96 Nonradioactive Cytotoxicity Assay kit [Promega] according to the manufacturer's specification). The results were average of three independent experiments performed in duplicate.

Horse Radish Peroxidase (HRP) assay

This assay was modified from(14). HeLa cells, in 6 well plates, were intoxicated with *P. aeruginosa* expressing ExoS derived constructs for 1 h at 37°C when cells were washed once with serum-free MEM, followed by the addition of 1 ml of pre-warmed serum-free MEM containing 2 mg/ml of HRP (Sigma) and 1% bovine serum albumin (BSA) to each well. HRP uptake was conducted at 37°C for 40 min. The uptake was stopped by washing the cell monolayers five times with ice-cold PBS containing 1% BSA. After the final wash, cells were scraped into 1 ml of ice-cold PBS and pelleted at 800 × g for 3 min. The cell pellet was washed one more time by suspension in 1 ml of ice-cold PBS followed by centrifugation. The final cell pellet was suspended in 0.5 ml of cold PBS and divided into two equal vials. One vial was lysed with 0.1% Triton X-100 to release intracellular HRP, while the other vial was not lysed and used as a background control for the detection of residual extra cellular HRP. This was subtracted from the corresponding sample lysed with Triton. The lysate was assayed for HRP activity in a 96-well microplate (Costar Co.) using ULTRA-TMB (Thermo Scientific) as substrate. The reaction was conducted at RT for 5–30 min and stopped by adding 100 μl of 0.1 N H_2SO_4. The products were quantified by measuring absorption at 450 nm in a Bio-Rad microplate reader (Victor 3). The invasion assays were normalized to the number of cells in each well, as determined by measuring the total amount of lactate dehydrogenase, using the

CytoTox 96 Nonradioactive Cytotoxicity Assay kit [Promega]. Results were normalized to GAPDH and were reported as the average of at least three independent experiments performed in duplicate.

EGF-activated EGFR degradation

HeLa cells were intoxicated with *P. aeruginosa* expressing ExoS derived constructs with synchronization for 1 h, and incubated with or without 100 ng/ml of EGF in serum free MEM for 1 hr. Cells were then washed, lysed and analyzed by SDS-PAGE and Western blotting with sheep anti-EGFR antibody (Santa Cruz Biotech). The amount of EGFR degraded was expressed as a percentage of total EGFR present before induction. GAPDH was used as a negative control. The mean and SD from three independent experiments were shown.

Immunofluorescence microscopy

P. aeruginosa intoxicated HeLa cells were permeablized with 0.1% Triton X-100 in 4% formaldehyde for 15 min at RT, followed by 3 washes with PBS. Cells were blocked with 1 % BSA in PBS for 20 min at RT then incubated with individual primary antibodies. ExoS contained a C-terminal HA epitope that was visualized with mouse α-HA IgG (1:1,000, 1 hr at RT), cells were washed three times with PBS and incubation with goat α-mouse IgG-alexa-568 (1:500). Cells were washed again, mounted and observed by Leica confocal scanning microscopy (Leica, 100 X oil objective). Plasma membrane was visualized with Image-iT™ LIVE Plasma Membrane and Nuclear Labeling Kit (Molecular Probes) according to the manufacturer, using Alexa594 WGA.

Image quantification

Fluorescent signal was analyzed by “line scan” in MetaMorph software (Molecular Devices). Correlation efficiency (Intensity Correlation quotient, ICQ) of fluorescence was determined using ImageJ Plugin intensity correlation analysis as described in(47,48) and are the average of scores from > 20 cells. Scale of ICQ is: −0.5~0.5, positive values indicate colocalization; 0 value indicate independent interactions; and negative values indicate segmentation of events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and nomenclature

P. aeruginosa
: *Pseudomonas aeruginosa*

G
: RhoGAP, Rho GTPase Activating Protein

A
: ADPr, ADP-ribosyltransferase

HRP
: Horseradish peroxidase

EGF

epidermal growth factor

EGFR

epidermal growth factor receptor

CCV

clathrin coated vesicles

EE

early endosomes

TfnR

transferrin receptor

EEA1

early endosome antigen 1

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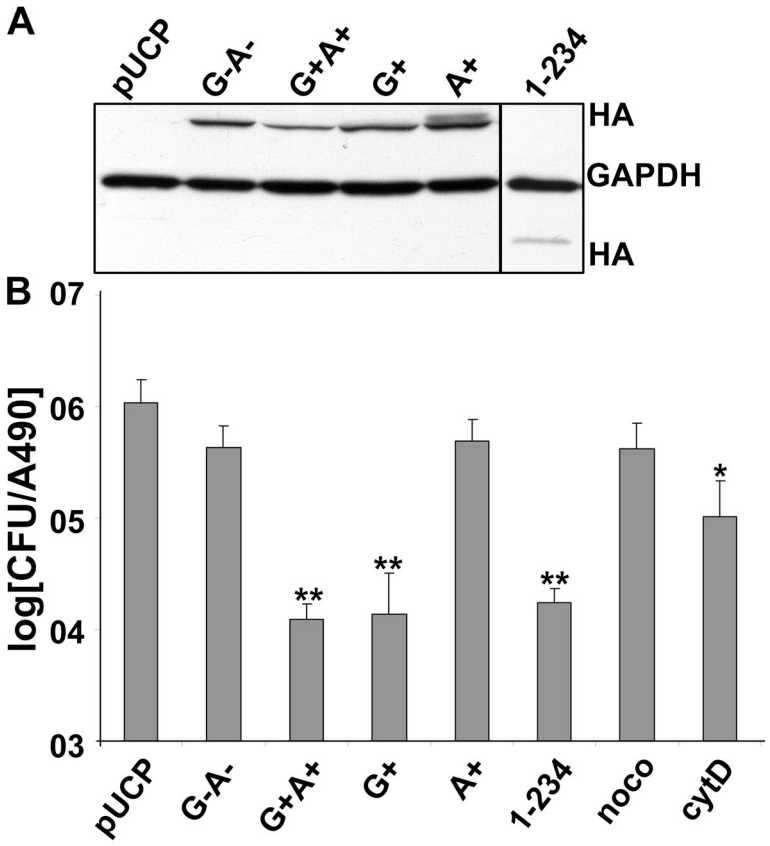

Figure 1. *P. aeruginosa* ExoS inhibition of bacterial internalization by HeLa cells is RhoGAP dependent

HeLa cells were intoxicated with *P. aeruginosa* expressing ExoS derived constructs. A) Three hr post intoxication (pi), cells were collected, lysed in SDS-PAGE sample buffer, and subjected to SDS-PAGE and immunoblotted for HA, using Super Signal detection. GAPDH was also probed as a loading control. B) Three hr pi, cells were incubated with 300 μg/ml of Gentamycin for 1 hr to kill extracellular bacteria. Cells were then collected, washed and lysed in 1% Triton X-100 to release intracellular bacteria. Lysates were diluted serially, plated on LB agar to quantify the CFU of internalized bacteria. HeLa cells were pretreated with nocodazole (noco) or cytochalasin D (cytoD) for 0.5 hr, then infected with catalytic null pUCP-ExoS(R146K, E381/379D)-HA (G^-A^-) as negative or positive controls, respectively. Results were normalized with total cell number by LDH activity and reported as the average of three independent experiments. Constructs used: pUCP vector control (pUCP), catalytic null pUCP-ExoS(R146K, E381/379D)-HA (G^-A^-), wild type pUCP-ExoS-HA (G^+A^+), GAP only pUCP-ExoS-E381D-HA (G^+), ADPr only pUCP-ExoS-R146K-HA (A^+), truncated ExoS with GAP only pUCP-ExoS-1-234-HA, *, $p<0.05$, **, $p<0.01$.

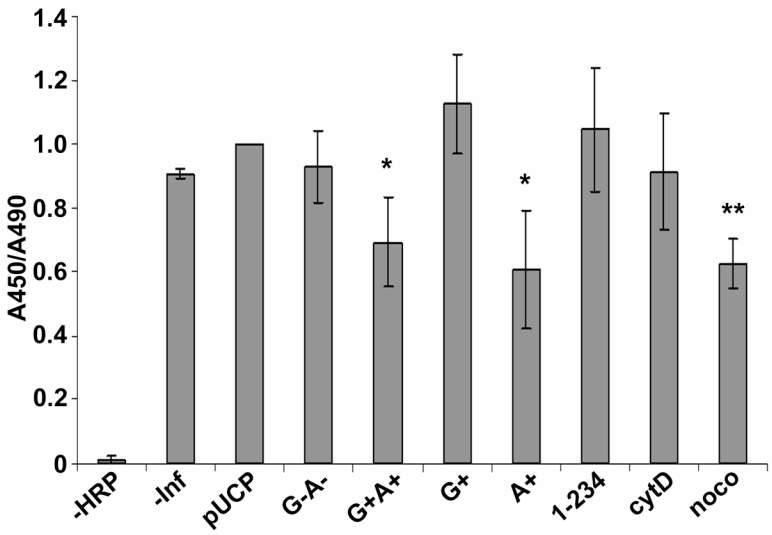

Figure 2. *P. aeruginosa* ExoS ADPr inhibits HRP uptake

HeLa cells were intoxicated with *P. aeruginosa* expressing ExoS derived constructs with synchronization or left uninfected (-inf). One hr pi, cells were incubated with 5mg/ml of HRP for 40 min. Cells were washed and lysed in 1% triton X-100 to release intracellular HRP. HPR activity was measured using Ultra-TMB substrates in ELISA plates. HeLa cells were pretreated with nocodazole (noco) or cytochalasin D (cytoD) for 0.5 hr, then infected with catalytic null pUCP-ExoS(R146K, E379/381D)-HA (KDD) as negative or positive controls. Results were normalized with total cell number by LDH activity. Value in KDD sample was normalized to one in each experiment. Constructs used were as described in Figure 1 *, $p<0.01$, ** $p<0.001$.

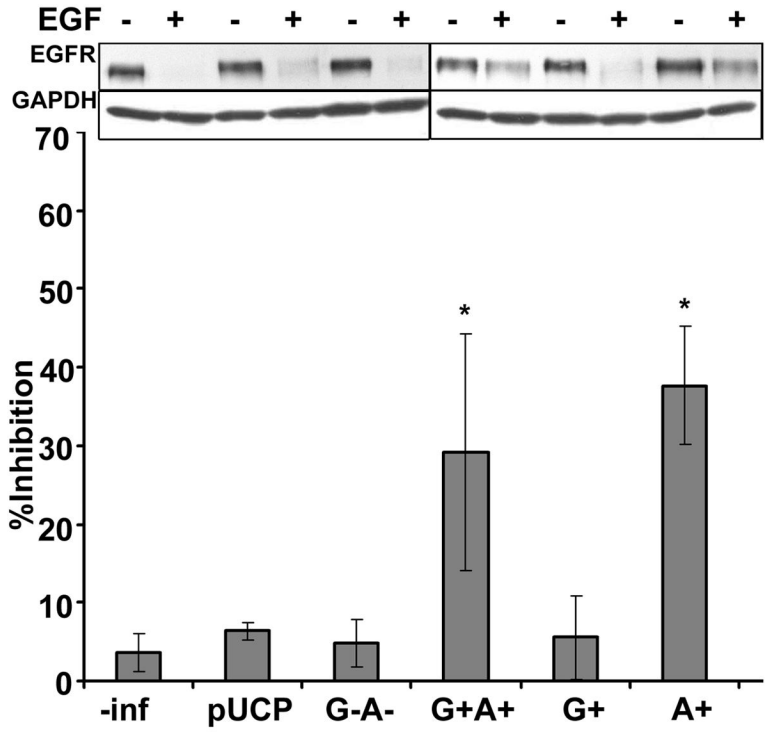

Figure 3. *P. aeruginosa* ExoS ADPr inhibits EGF-activated EGFR degradation

HeLa cells were intoxicated with *P. aeruginosa* expressing ExoS derived constructs with synchronization or left uninfected (-inf). One hr pi, cells were stimulated with 100 ng/ml of EGF for 1 hr. Cells were then collected, lysed in SDS-PAGE sample buffer, and subjected to 8% SDS-PAGE and immunoblotted for EGF receptor (EGFR) using Super Signal. GAPDH (GDH) was also probed as a loading control. HeLa cells were pretreated with nocodazole (noco) or cytochalasin D (cytoD) for 0.5 hr, then infected with catalytic null pUCP-ExoS(R146K, E381/379D)-HA (KDD) as negative or positive controls. A representative immunoblot is shown in the upper panel. Percentage of inhibition was quantified by densitometry of immunoblot. Results were normalized with total cell protein by GAPDH. Shown are averages of three independent experiments. Constructs used were as described in Figure 1. *, $p<0.01$.

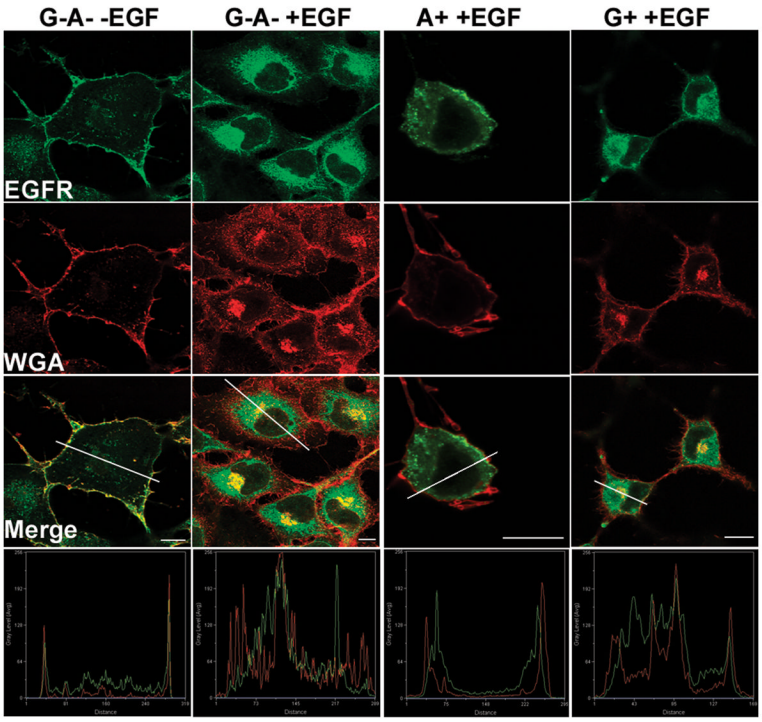

Figure 4. *P. aeruginosa* ExoS ADPr does not inhibit EGF-activated EGFR internalization

HeLa cells were intoxicated with *P. aeruginosa* expressing ExoS derived constructs with synchronization. One hr pi, cells were stimulated with 100 ng/ml of EGF for 1 hr. Cells were washed, fixed and endogenous EGF receptor (EGFR) was visualized by immunostaining using sheep anti-EFGR as primary antibody followed by alexa488 rabbit anti-sheep secondary antibody. Alexa 594-Wheat germ agglutinin (WGA) was added 20 min prior to fixation to label plasma membrane. Images were taken under a Leica confocal scanning microscope with a 100× oil objective. Fluorescence intensity along the line in each merge image was shown at the bottom. Constructs used were as described in Figure 1. Bar: 10nm.

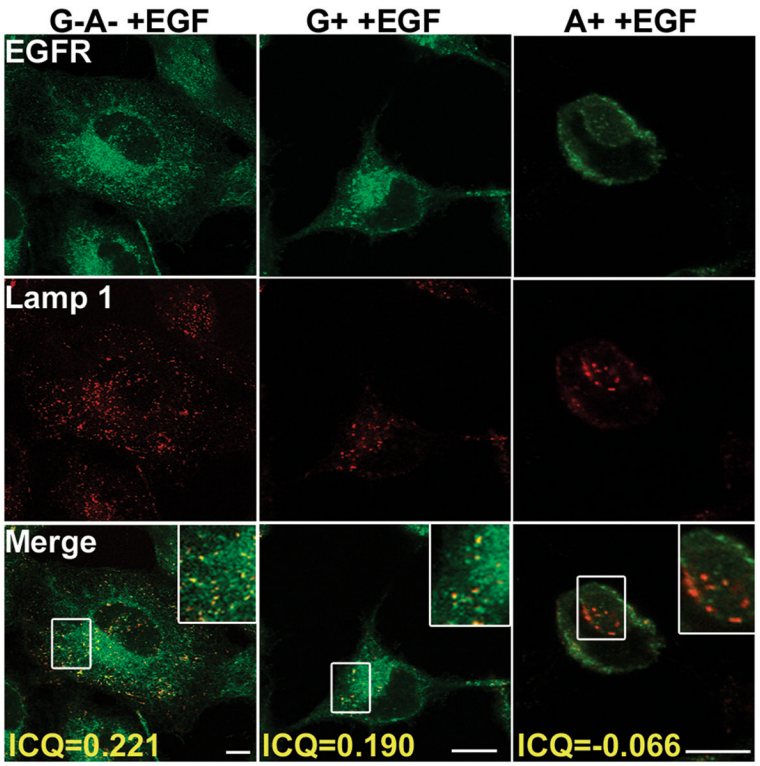

Figure 5. *P. aeruginosa* ExoS ADPr inhibits EGF-activated EGFR trafficking to the lysosome

HeLa cells were intoxicated with *P. aeruginosa* expressing ExoS derived constructs and treated with EGF as described in Figure 4. Cells were washed, fixed, and endogenous EGFR was visualized by immunostaining using sheep anti-EFGR as the primary antibody followed by alexa488 rabbit anti-sheep secondary antibody. Endogenous Lamp 1 was visualized by immunostaining using mouse anti-Lamp 1 as the primary antibody followed by alexa568 mouse anti-rabbit secondary antibody. Images were taken under a Leica confocal scanning microscope with a 100× oil objective. Bar: 10nm. Correlation of green and red signals was displayed as ICQ value calculated as indicated in Materials and Methods.

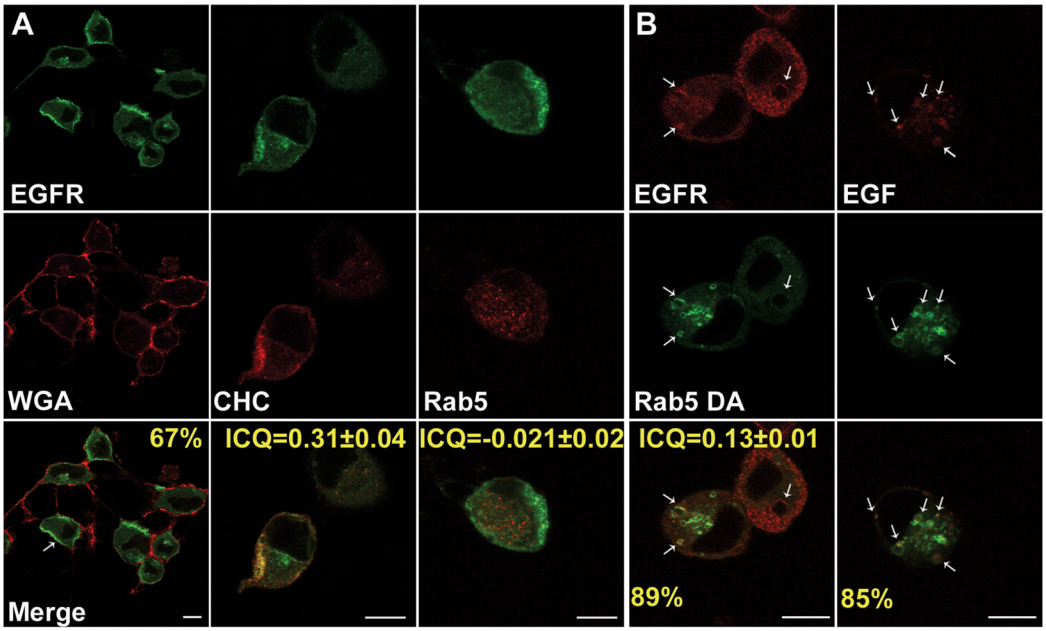

Figure 6. DA-Rab5 rescues *P. aeruginosa* ExoS ADPr inhibition of EGF-activated EGFR trafficking in the early endosome

A) HeLa cells were intoxicated with *P. aeruginosa* PA103, Δ*exo*U, *exo*T::Tc carrying pUCP-ExoS-R146K-HA (A^+) with synchronization. One and ½ hr pi, cells were stimulated with 100 ng/ml EGF for 30 min. Cells were washed, fixed and endogenous EGF receptor (EGFR) was visualized by immunostaining using sheep anti-EFGR as primary antibody followed by alexa488 rabbit anti-sheep secondary antibody. Endogenous clathrin heavy chain (CHC), and Rab 5 was visualized by immunostaining using mouse anti-CHC or Rab 5 as primary antibody followed by alexa568 conjugated secondary antibody. Arrows: cells with EGF receptor accumulated in cell periphery. B) HeLa cells were transfected with constitutively active Rab 5 fused with green fluorescent protein (GFP-Rab5-DA) and infected with *P. aeruginosa* PA103, Δ*exo*U, *exo*T::Tc carrying pUCP-ExoS-R146K-HA (A^+). One and ½ hr pi, (left) cells were treated with EGF for 30 min. Cells were then washed, fixed and endogenous EGF receptor (EGFR) was visualized as in A. (right) Alexa 568-EGF (5 mg/ml) was added to cells, fixed, and mounted. Images were taken under a Leica confocal scanning microscope with a 100× oil objective. Arrows: Rab 5 vesicles positive for EGFR staining or alexa-EGF. Bar: 10nm.

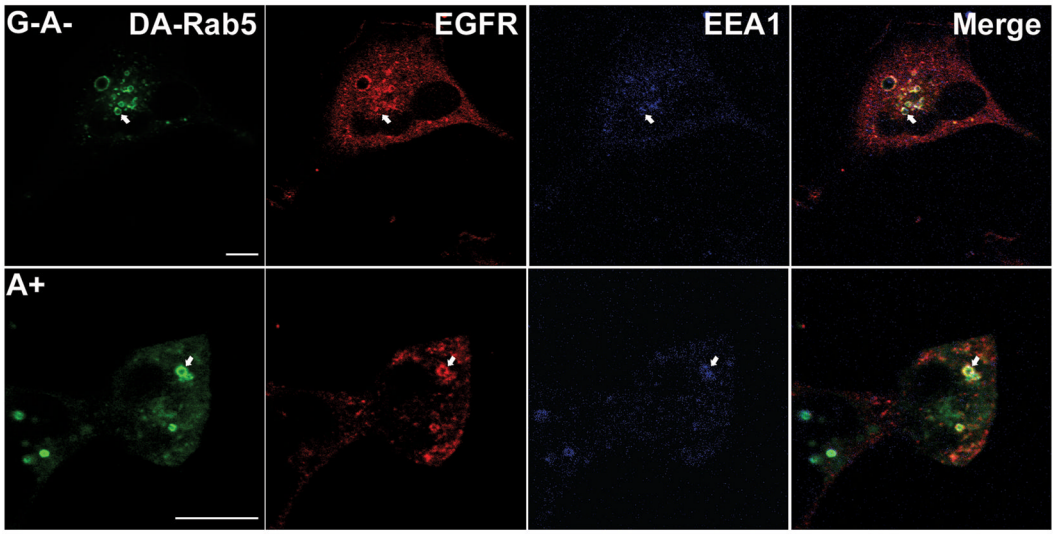

Figure 7. DA-Rab5 recruits EEA1 in ExoS A^+ intoxicated cells

HeLa cells transfected with Constitutively active Rab 5 fused with green fluorescent protein (GFP-Rab5-DA) were co-cultured with *P. aeruginosa* PA103, Δ*exo*U, *exo*T::Tc carrying pUCP-ExoS-KDD-HA (G^-A^-) or pUCP-ExoS-R146K-HA (G^+) with synchronization. One and ½ hr pi, cells were stimulated with 100 ng/ml EGF for 30 min. Cells were washed, fixed and endogenous EGF receptor (EGFR) was visualized by immunostaining, using sheep anti-EFGR as primary antibody followed by alexa568 rabbit anti-sheep secondary antibody. Endogenous early endosome antigen 1 (EEA1) was visualized by immunostaining using mouse anti-EEA1 as primary antibody followed by alexa633 conjugated secondary antibody. Images were taken with a Leica confocal scanning microscope using a 100× oil objective. Arrows: Rab 5 vesicles positive for both EGFR and EEA 1 staining. Bar =10nm.