**Pseudomonas aeruginosa** Cif Protein Enhances the Ubiquitination and Proteasomal Degradation of the Transporter Associated with Antigen Processing (TAP) and Reduces Major Histocompatibility Complex (MHC) Class I Antigen Presentation*

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### Background:

*P. aeruginosa* Cif degrades the ABC transporters CFTR and P-glycoprotein.

### Results:

Cif increases the ubiquitination and degradation of TAP1 and decreases MHC class I antigen presentation in airway epithelial cells.

### Conclusion:

Cif is the first bacterial factor identified that inhibits TAP function and MHC class I antigen presentation.

### Significance:

These observations suggest a mechanism whereby *Pseudomonas* infection increases the severity and duration of respiratory viral infections.

Cif (PA2934), a bacterial virulence factor secreted in outer membrane vesicles by *Pseudomonas aeruginosa*, increases the ubiquitination and lysosomal degradation of some, but not all, plasma membrane ATP-binding cassette transporters (ABC), including the cystic fibrosis transmembrane conductance regulator and P-glycoprotein. The goal of this study was to determine whether Cif enhances the ubiquitination and degradation of the transporter associated with antigen processing (TAP1 and TAP2), members of the ABC transporter family that play an essential role in antigen presentation and intracellular pathogen clearance. Cif selectively increased the amount of ubiquitinated TAP1 and increased its degradation in the proteasome of human airway epithelial cells. This effect of Cif was mediated by reducing USP10 deubiquitinating activity, resulting in increased polyubiquitination and proteasomal degradation of TAP1. The reduction in TAP1 abundance decreased peptide antigen translocation into the endoplasmic reticulum, an effect that resulted in reduced antigen available to MHC class I molecules for presentation at the plasma membrane of airway epithelial cells and recognition by CD8+ T cells. Cif is the first bacterial factor identified that inhibits TAP function and MHC class I antigen presentation.

### Previous work from our laboratory identified a secreted protein from *Pseudomonas aeruginosa*, Cif (PA2934), which selectively decreases the expression and function of some ABC transporters in the plasma membrane of human airway epithelial cells, including CFTR and P-glycoprotein, but not MRP1 or MRP2 (1–3). Cif is secreted from *P. aeruginosa* in bacterial-derived outer membrane vesicles (OMVs), which enhance the delivery of bacterial toxins across mucus plugs and over long distances in the airways and fuse with host cell lipid raft domains and thereby deliver Cif to the cytosol (4). Cif is highly expressed by clinical isolates of *P. aeruginosa* and is detected in sputum samples from patients with cystic fibrosis and pseudomononal pneumonia (2). In the current study, we tested the hypothesis that Cif regulates another ABC transporter, the transporter associated with antigen processing (TAP), which plays a key role in bacterial and viral antigen presentation via MHC class I molecules and pathogen clearance by CD8+ cytotoxic T cells.

Upon acute bacterial and viral infection, cytosolic pathogen proteins are processed by the proteasome, and peptide antigens are translocated via the TAP complex into the endoplasmic reticulum (ER) where they are loaded onto MHC class I molecules by chaperones. The peptide-loaded MHC class I molecules then traffic to the cell surface, where they present antigen to be recognized by epitope-specific CD8+ T cells, the activation of which is an important arm of the adaptive immune response. Because Cif induces the degradation of some, but not all, ABC proteins and because TAP1 and TAP2 are members of the ABC transporter family of transport proteins, we conducted a series of experiments to test the hypothesis that Cif directs the degradation of the TAP complex and thereby reduces antigen transport into the ER and the appearance of antigen-loaded MHC class I molecules at the plasma membrane. Our data...
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demonstrate that Cif increases the ubiquitination and proteasomal degradation of TAP1, but not TAP2, resulting in a reduction in antigen transport into the lumen of the ER, a reduction of antigen-loaded MHC class I molecules at the plasma membrane, and a dramatic decrease in, and recognition of, virus-infected cells by CD8+ T cells.

EXPERIMENTAL PROCEDURES

Cell Culture—The role of Cif in TAP peptide translocation was studied in human airway epithelial cells (CFBE41o- cells, homozygous for the ΔF508 mutation) stably expressing WT-CFTR (hereafter called airway epithelial cells), described in detail by several laboratories (6, 7) and A549 alveolar epithelial cells expressing WT-CFTR. Airway epithelial cells and A549 cells were grown and polarized in an air-liquid interface culture at 37 °C for 6–9 days, as described (6).

Pseudomonas aeruginosa Cultures and OMV Purification—Lysozyme broth (LB) was inoculated with P. aeruginosa strain UCBPP-PA14 (PA14) (8), and cultures were prepared as reported previously (2). P. aeruginosa OMVs (+Cif) OMVs and (−Cif) OMVs were isolated from an overnight culture and diluted to approximate the OMVs produced by 108 bacteria. A bacterial count of 103 to 1010 is relevant because this is the bacterial density often detected in cystic fibrosis patient sputum samples (9). OMVs were purified using a differential centrifugation and discontinuous OptiPrep gradient protocol adapted from Bauman and Kuehn (10).

Peptide Translocation Assays—Peptide translocation was measured as described previously using 125I-labeled B27-3 glyccopeptide-1 and ER microsomes prepared from (+Cif) OMV- or (−Cif) OMV-treated polarized airway epithelial cells (11).

Antigen Presentation Assays—Presentation of the SIINFEKL peptide was assessed using EL-4 cells treated in the presence or absence of P. aeruginosa (+Cif) OMVs or (−Cif) OMVs, then inoculated with PR8/SIINFEKL influenza at a multiplicity of infection of 10 and processed for flow cytometry by indirect labeling using the 25.D1-16 clone antibody to recognize specific Kb-SIINFEKL complexes (12–14). Untreated cells were exposed in parallel with 1 nM OVA peptide as a positive control for 25.D1-16 clone antibody labeling. MHC class I was detected with an antigen-presenting cell (APC)-conjugated H-2Kb antibody (Lifespan Biosciences).

CD8+ T Cell Activation Assays—MLE-Ko cells were infected with influenza A/Japan/57 or influenza A/PR8 in the continuous presence of (+Cif) OMVs or (−Cif) OMVs. The T cell line 40-2, which specifically recognizes the influenza A/Japan/57 HA201–212 peptide in the context of MHC I Kd, was added to infected MLE-Ko cells at an effector:target ratio of 5:1. Supernatants were assayed for the presence of TNF-α using the mouse TNF-α OptEIA ELISA set (BD Biosciences) according to the manufacturer’s instructions.

Identification of Active Deubiquitinating Enzymes (DUBs)—To identify active DUBs in the ER of airway epithelial cells we used a chemical probe screening approach designed and described in detail by Dr. Hidde Ploegh (15–17) and recently published by our laboratory (18). The specificity of the HA-UbVME probe for active DUBs was confirmed with the addition of N-ethylmaleimide (10 mM), which inhibits cysteine protease DUBs, during the labeling reaction (15–17). Airway epithelial cells were lysed in radioimmunoprecipitation assay buffer (25 mM Tris-HCl, pH 7.6, 10 mM NaCl, 1% Nonidet P-40 (IGEPAL), 1% sodium deoxycholate, 0.1% SDS), and 0.1 μg of the HA-UbVME probe was added to 20 μg of ER microsomal fractions (ER was isolated as described below). The HA-UbVME probe forms an irreversible, covalent bond with active DUBs. DUBs covalently linked to the HA-UbVME probe were identified by immunoprecipitation of the HA-UbVME-DUB complex(s) from purified ER fractions of airway epithelial cells using 5 μg of anti-HA monoclonal antibody (Santa Cruz Biotechnology) complexed to protein A-agarose (19). Immunoprecipitated proteins were eluted from the protein A-agarose complex(s) in Laemmli sample buffer (Bio-Rad) containing 80 mM dithiothreitol. Immunoprecipitated proteins were separated by SDS-PAGE using 7.5% Tris-HCl gels (Bio-Rad) and analyzed by Western blotting. In the figures the lysate lane represents 5% of total cell lysate. The remainder of the lysate was used for immunoprecipitation experiments. All immunoprecipitation experiments are quantified by normalizing the co-immunoprecipitated signal with that of the protein used to pull down, thereby normalizing for the efficiency of the pulldown.

Isolation of Endoplasmic Reticulum—To assess the trafficking of Cif through the retrograde transport pathway, we used differential centrifugation and OptiPrep continuous gradients to separate the plasma membrane, Golgi apparatus, and ER fractions from airway epithelial cells, a protocol adapted from a previous study by Yang et al. (20). Plasma membrane (Na+,K+-ATPase), Golgi apparatus (TGN58), and ER (calnexin, ERP57) resident proteins were used to identify these compartments in the fractionations to identify the localization of Cif.

Ubiquitination Assay—To assess the amount of ubiquitinated TAP in airway epithelial cells, a protocol was adapted from Urbe et al. (21) and recently published by our laboratory (18). Briefly, polarized airway epithelial cells were treated with P. aeruginosa OMVs as noted in figure legends in the presence of 37.5 μM MG132 to prevent degradation of ubiquitinated proteins. Cells were then lysed in boiling lysis buffer (2% SDS, 1 mM EDTA, 50 mM sodium fluoride, and Complete Protease Inhibitor Mixture (Roche Diagnostics)) preheated to 100 °C. Lysates were transferred to screw-cap tubes and incubated for 10 min at 100 °C, then cooled to room temperature before the lysates were diluted by the addition of 4 volumes of the dilution buffer (2.5% Triton X-100, 12.5 mM Tris, pH 7.5, 187.5 mM NaCl, and Complete Protease Inhibitor Mixture). Cell debris was pelleted with low speed centrifugation (3000 × g), and lysates were immunoprecipitated overnight at 4 °C with 5 μg of anti-TAP1 antibody (gift from Dr. Naveen Bangia) complexed with protein G-agarose. Immunoprecipitated complexes were washed three times with dilution buffer (2% Triton X-100, 0.4% SDS, 10 mM Tris, pH 7.5, 150 mM NaCl), once with a high salt wash buffer (200 mM NaCl, 400 mM NaOAc), and once more with the dilution buffer before analysis with SDS-PAGE and Western blotting using a ubiquitin antibody that recognizes only polyubiquitin addition (FK1 ubiquitin clone; BioMol). The quantification of polyubiquitinated TAP1 was calculated as the signal obtained with the ubiquitin antibody (blotting immunoprecipitated TAP1) nor-
malized for immunoprecipitated TAP1, detected in Western blots with the TAP1 antibody and expressed as percentage of (+Cif) OMV-treated samples.

**Western Blot Analysis**—Western blot analysis was performed as described previously (22). Samples were prepared in Laemmli sample buffer (Bio-Rad) containing 80 mM dithiothreitol and resolved on 7.5% Tris-HCl SDS-polyacrylamide gels (Bio-Rad). Western blot experiments are normalized for ezrin in the cell lysate as a loading control and when indicated, expressed as percentage of the control treatment. Protein abundance in Western blots was measured using ECL detection substrate (Pierce), the film was scanned using an Epson high resolution scanner, and images were quantified using ImageJ 64 (National Institutes of Health).

**Plasmids and Transient Transfections**—Plasmids containing GFP-WT-USP10 and GFP-USP10 (C424A) were a generous gift from Dr. Susanna Chiocca (European Institute of Oncology, IFOM-IEO campus (23)). Transient transfections of airway epithelial cells with GFP-WT-USP10 and GFP-USP10-C424A were conducted using Effectene (Qiagen) according to the manufacturer’s instructions.

**RNA-mediated Interference**—Ubiquitin-specific protease-10 (USP10) abundance was selectively reduced using siRNA purchased from Qiagen, by methods described previously (22). In brief, airway epithelial cells were seeded at 0.1 × 10^6 on 24-mm Transwell permeable membrane supports, and on day 4 after seeding, and cells were transfected with HiPerfect transfection reagent according to the manufacturer’s protocol (Qiagen). Sequences for siRNAs are: siUSP10 sense, 5’-CACAGCUCCUGUGACUCUTT-3’, and siNegative scrambled sense, 5’-UUC-UCCGAACGUGUCACGU-3’. Cells were studied on day 8 after seeding (i.e. 4 days after transfection with siRNA).

FIGURE 1. *P. aeruginosa* Cif induces the degradation of TAP1. a. OMVs isolated from Cif-expressing *P. aeruginosa* (subsequently called (+Cif) OMVs) applied apically to polarized airway epithelial cells induce a time-dependent reduction in TAP1, but not TAP2, protein abundance, as assessed by Western blot analysis. Ezrin is a protein-loading control, and TAP data for each lane are normalized for ezrin abundance and presented as a percentage of control (0 min, no OMV treatment). Similar results were obtained with Cif in AS49 cells (data not shown). b. (-Cif) OMVs from *P. aeruginosa* do not reduce TAP1 protein levels. (+Cif) OMVs and (-Cif) OMVs were applied to the apical side of airway epithelial cells, and TAP1 protein abundance was assessed by Western blot analysis. Both representative blots shown were probed for TAP1. Quantification for Western blot experiments is presented below representative blots. c. Recombinant Cif protein reduces TAP1 protein levels. Recombinant Cif protein (50 μg) was applied apically to airway epithelial cells for various times and TAP protein abundance assessed by Western blot analysis. Ezrin was used as a loading control. Quantification for Western blot experiments is found below representative blots. d. Cif reduces TAP1 abundance in a dose-dependent manner. Recombinant Cif protein was applied to the apical side of airway epithelial cells at various doses for 90 min, and TAP1 protein abundance was assessed by Western blot analysis. Quantification for Western blot experiments is presented below representative blots. Data are expressed as the mean ± S.E. (error bars), n = 3. *, p < 0.05.
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**RESULTS**

**Cif Reduces TAP1, but Not TAP2, Abundance**—*P. aeruginosa* utilizes OMVs to deliver toxins (including Cif) to host airway epithelial cells, especially across barriers like the mucus layer in the lung (4). For this reason, we employed *P. aeruginosa* OMVs from a wild-type strain of *P. aeruginosa* (denoted subsequently as (+Cif) OMVs) to deliver Cif to polarized human airway epithelial cells. (+Cif) OMVs reduced TAP1, but not TAP2, protein abundance in a time-dependent manner (Fig. 1a). Importantly, OMVs from a *P. aeruginosa* strain in which Cif had been deleted (denoted subsequently as (−Cif) OMVs) did not alter TAP1 abundance (Fig. 1b), revealing that Cif is necessary for reducing TAP1 abundance. These results were confirmed in A549 cells, in which (+Cif) OMVs reduced TAP1 abundance by 57.5 ± 2.8% compared with A549 cells treated with (−Cif) OMVs. Recombinant Cif protein applied to the apical membrane of polarized airway epithelial cells also reduced TAP1 but not TAP2 protein abundance (Fig. 1c) in a concentration-dependent manner (Fig. 1d), revealing that Cif alone is sufficient to reduce TAP1 abundance.

**Cif Inhibits TAP-mediated Peptide Transport into the ER and Cell Surface MHC Class I Antigen Presentation**—To determine whether Cif, by decreasing TAP1 abundance, reduced TAP-mediated transport of peptides into the ER, studies were conducted to measure peptide translocation into the ER by measuring 125I-labeled peptide translocation into ER microsomes of airway epithelial cells, as described (11). (+Cif) OMVs reduced peptide uptake by 61 ± 7%, compared with peptide uptake in cells treated with (−Cif) OMVs (Fig. 2a). The decrease in peptide transport of 61 ± 7% measured 90 min after addition of (+Cif) OMVs, but not after addition of (−Cif) OMVs, was similar to the 65 ± 3.5% reduction in TAP1 protein abundance measured at the same time point (Fig. 1a).

We next sought to assess whether the Cif-mediated reduction in peptide translocation into the ER had a physiological consequence in viral antigen presentation or CD8+ T cell recognition of virus-infected cells. To determine whether Cif alters the expression of specific, peptide-loaded MHC class I molecules at the cell surface, we examined MHC class I antigen presentation of the OVA minimal epitope, SIINFEKL using a specific antibody (clone 25.D1-16 (24, 25)) to detect Kb-SIINFEKL complexes at the cell surface in (−Cif) OMV- and (+Cif)
OMV-treated EL-4 cells. In this experiment, SIINFEKL was expressed by a recombinant PR8 influenza and therefore processed endogenously in the context of a bona fide virus infection. (+Cif) OMVs reduced the cell surface expression of Kb-SIINFEKL complexes by 91.8%, whereas (-Cif) OMVs had no significant effect on antigen presentation (Fig. 2b).

To determine whether Cif inhibited CD8$^+$ T cell recognition of influenza A virus-infected epithelial cells, we compared the effect of (+Cif) OMVs and (-Cif) OMVs on CD8$^+$ T cell recognition of virus-infected epithelial cells. We infected MLE-Kd epithelial cells with A/Japan/57 influenza virus and assessed recognition by a CD8$^+$ T cell line specific for a Kd-restricted epitope of A/Japan/57 hemagglutinin (HA204–212). CD8$^+$ T cell recognition and activation were assessed by measuring TNF-α production by ELISA. As shown in Fig. 2d, CD8$^+$ T cell TNF-α production resulting from recognition of influenza virus-infected epithelial cells treated with (+Cif) OMVs was reduced by 81 ± 2% compared with recognition of infected cells treated with (-Cif) OMVs. An unrelated strain of influenza A virus, A/PR8/34 (PR8), which does not contain a cross-reactive epitope recognized by this T cell line, served as a negative control (Fig. 2d). Thus, the P. aeruginosa-secreted toxin Cif disrupts viral antigen presentation and CD8$^+$ T cell recognition of influenza A-infected epithelial cells and neither was impacted by OMV isolated

**FIGURE 3.** **Cif trafficking to the ER via the retrograde pathway is required to reduce TAP1 abundance.** Airway epithelial cells were lysed, and intracellular organelles were prepared via differential centrifugation and OptiPrep gradient separation. a, isolation of intracellular organelles. Plasma membrane (Na$^+$,K$^+$-ATPase), Golgi apparatus (TGN58), and ER (calnexin) resident proteins were used to identify by Western blot analysis the plasma membrane, Golgi apparatus, and ER, respectively. b, OptiPrep gradient fractionation and differential centrifugation analysis of Cif (delivered via (+Cif) OMVs) at various times after incubation with airway epithelial cells reveals the sequential trafficking of Cif from the plasma membrane, to the Golgi, and then to the ER. Fractions correspond to those in a. Western blot analysis was performed for Cif. c, Cif protein detected in each fraction is quantified. Data are presented as percentage of Cif detected in each fraction compared with the total Cif detected in all fractions. d, inhibition of retrograde transport to the ER with brefeldin A (BrefA; 100 μM) inhibits the Cif-mediated decrease in TAP1 protein abundance, as assessed by Western blot analysis. n = 3; *, p < 0.05.
from *P. aeruginosa* in which the Cif gene had been deleted. Taken together these studies support the conclusion that Cif is necessary and sufficient to reduce TAP1 protein abundance in airway epithelial cells and that this decrease in TAP1 significantly reduces TAP-mediated peptide translocation into the ER and subsequent MHC class I-mediated antigen presentation at the cell surface.

Retrograde Trafficking of Cif to the ER Is Required for the Cif-mediated Reduction in TAP1—Because TAP1 is resident in the ER, the observation that Cif reduced TAP1 abundance suggests that Cif traffics from the plasma membrane where OMVs fuse with lipid rafts (4) and proceeds through the retrograde pathway to the ER. Thus, two studies were conducted to determine whether Cif traffics to the ER via the retrograde pathway and whether this trafficking is required for Cif activity. First, we isolated subcellular compartments to track the movement of Cif through the retrograde pathway from the plasma membrane, through the Golgi, and to the ER. Using differential centrifugation and gradient fractionation, we employed a technique that separates the plasma membrane, trans-Golgi network and ER (Fig. 3a). Fig. 3, b and c, demonstrates that Cif, delivered to airway epithelial cells in (+Cif) OMVs, traffics through the retrograde pathway with a time course that is consistent with other bacterial proteins, like AB toxins, that traffic through the retrograde pathway in host cells (26). Second, if Cif is required to reach ER-associated membranes to alter TAP1 abundance, inhibiting the retrograde transport of Cif should block the effect of Cif on TAP1. Inhibition of retrograde transport with brefeldin A for 90 min eliminated the effect of Cif on TAP1 abundance, as measured by Western blot analysis (Fig. 3d). Taken together, both studies revealed that Cif traffics in the retrograde pathway to ER-associated membranes and that this trafficking is required for Cif to reduce TAP1 abundance.
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Cif Enhances the Ubiquitination of TAP1—Next, studies were conducted to test the hypothesis that Cif reduces the abundance of TAP1 by enhancing its ubiquitination and subsequent degradation by the proteasome. (+Cif) OMVs, but not (−Cif) OMVs, increased the amount of polyubiquitinated TAP1 in human airway epithelial cells by 142 ± 12% (Fig. 4, a and b). The ubiquitination status of TAP1 was quantified by normalizing the ubiquitination signal from the Western blot of immunoprecipitated TAP1 probed with ubiquitin, to TAP1 measured in Western blots of cell lysates and then expressed as a percentage of the control. The Cif-induced reduction in TAP1 abundance was blocked by the proteasomal inhibitors lactacycstin or MG132, but not by the lysosomal inhibitors chloroquine and ammonium chloride (Fig. 4c). It is important to note that Cif did not affect the total amount of polyubiquitinated proteins (Fig. 4a). Because these experiments were conducted in the presence of cycloheximide to block protein synthesis, these results demonstrate that Cif enhances the degradation of TAP1 by increasing the amount of polyubiquitinated TAP1 and its subsequent degradation in the proteasome.

Cif Inactivates the Deubiquitinating Enzyme USP10—Cif may increase the amount of polyubiquitinated TAP1, and thereby its degradation, by activating an E3 ligase and/or by inactivating a DUB. In a previous study we reported that Cif inhibits USP10 (27) activity. To determine whether Cif inhibits USP10 activity and thereby increases the amount of polyubiquitinated TAP1, we used a DUB activity assay to identify active DUBs associated with the ER of airway epithelial cells (16–18, 28). The DUB activity assay employs a HA-UbVME probe that forms an irreversible, covalent bond only with active DUBs. We isolated ER membranes (as demonstrated in Figs. 3a and 5a) using OptiPrep gradients, as described previously, that were not contaminated with Golgi membranes (i.e. absence of TGN58) or plasma membrane (i.e. absence of Na⁺,K⁺-ATPase) ((20), Fig. 3a). Using this assay, we identified several active DUBs in an isolated ER fraction (fraction 6), including USP10 (Fig. 5a). (+Cif) OMVs reduced the activity of USP10 by 51 ± 6%, without affecting the total abundance of USP10 in the ER fraction (Fig. 5b). Cif did not alter the activity of other ER-resident DUBs, including USP4, demonstrating the specificity of Cif for USP10 (Fig. 5b). These results are consistent with the conclusion that Cif inhibits USP10 DUB activity, which results in an increase in the amount of polyubiquitinated TAP1 and its degradation in the proteasome.

USP10 Regulates the Amount of Ubiquitinated TAP1 and TAP1 Abundance—Four sets of studies were conducted to demonstrate that USP10 regulates the amount of ubiquitinated TAP1 and TAP1 abundance. siRNA knockdown of USP10, in the absence of Cif, reduced USP10 protein abundance by 72 ± 3% (Fig. 6a) and increased the amount of ubiquitinated TAP1 by 169 ± 16% (Fig. 6, b and c), a level equivalent to the amount of ubiquitinated TAP1 produced by Cif (Fig. 6c). siRNA knockdown of USP10 also resulted in a similar reduction in TAP1 abundance compared with (+Cif) OMV treatment (Fig. 6d). Furthermore, addition of (+Cif) OMVs to cells in which USP10 was reduced by siUSP10 had no effect on TAP1 abundance (Fig. 6d). Moreover, transfection of wild-type USP10 increased TAP1 protein abundance (Fig. 6e), whereas expression of a dominant negative USP10 (USP10-C424A) decreased TAP1 abundance (Fig. 6e). These results demonstrate that USP10 regulates the amount of ubiquitinated TAP1 and, taken together with studies above, are consistent with the conclusion that Cif decreases TAP1 abundance, at least in part, by inhibiting USP10, thereby increasing the amount of polyubiquitinated TAP1 and its degradation in the proteasome.

DISCUSSION

Taken together with recent publications from our laboratory (4,7) our data herein reveal that the Cif virulence factor,
secreted in OMVs by *P. aeruginosa*, enters human airway epithelial cells via lipid rafts and travels via the retrograde pathway to reach the endoplasmic reticulum (4). The transit of Cif through the retrograde pathway is on the cytoplasmic face of transport vesicles because proteinase K, which does not degrade luminal endosomal proteins but can degrade proteins on the cytoplasmic face of endosomes, eliminated Cif from endosomal vesicles (4). In association with the cytoplasmic face of the endoplasmic reticulum, Cif inhibits USP10 activity which results in an increase in the amount of polyubiquitinated TAP1 and thus its proteasomal degradation, leading to a reduction of antigen translocation into the ER, and thereby reduced MHC class I antigen presentation at the plasma membrane.

The TAP transporter comprises two ABC half-transporters, TAP1 and TAP2, which form a heterodimeric complex that transfers peptides into the ER lumen by simultaneously binding peptide and ATP (29–33). Other members of the peptide-loading complex (PLC), in addition to TAP1 and 2, include the chaperone calreticulin, the thiol-oxidoreductase ERp57, and tapasin, which optimizes loading for stable binding of peptides (34–37). MHC class I molecules are also associated with TAP in the PLC (38, 39), and MHC class I molecules may associate with calnexin and ERp57 prior to their integration into the PLC (39). Thus, subcomplexes of the PLC exist within cells, but their roles in antigen presentation are not well understood (38). Efficient loading of high affinity peptides onto MHC class I molecules requires an appropriate stoichiometry of the PLC, which is critical for cell surface antigen presentation by MHC class I (40). Following peptide loading, MHC class I molecules are then transported to the trans-Golgi network for processing and sub-

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**FIGURE 6.** *USP10 regulates the amount of ubiquitinated TAP1 and TAP1 abundance.*

**a**, siRNA (15 nM) knockdown of USP10 in airway epithelial cells, as assessed by Western blot analysis for USP10 and normalized for ezrin is shown. Representative blots are presented. *b*, siRNA knockdown of USP10 increased the amount of ubiquitinated TAP1, as assessed by immunoprecipitation (IP) of TAP1 and Western blotting for polyubiquitinated TAP1. The amount of ubiquitinated TAP1 was similar in cells treated with (+Cif) OMVs and siUSP10. Polyubiquitin adducts are detected with the FK1 ubiquitin antibody. *siNeg* is a scrambled, negative control. Black boxes highlight ubiquitinated TAP1. *IgG*, immunoprecipitation using a nonimmune IgG was used as a specificity control. *Lysate lane* represents 5% of total cell lysate. The remainder of the lysate was used for immunoprecipitation experiments. All samples were run on the same gel, but the gel images were cut for presentation. *c*, quantification of Western blotting experiments is presented with representative blots. Ubiquitinated TAP1 is normalized to immunoprecipitated TAP1 and expressed as a percentage of the control (+Cif) OMV sample. *d*, siRNA knockdown of USP10 (labeled siUSP10) reduced protein abundance of TAP1 to a similar extent as (+Cif) OMV treatment (labeled siNeg + Cif). When USP10 abundance was reduced with siUSP10, (+Cif) OMVs had no effect on TAP1 abundance (compare siUSP10 with siUSP10 + Cif). Ezrin was used as a loading control. Quantification of Western blot experiments is presented below representative blots. *e*, overexpression of wild-type USP10 increased TAP1 protein abundance, whereas expression of a dominant negative USP10 (USP10-C424A) reduced the protein abundance of TAP1. Quantification for Western blot experiments is presented with representative blots. Data are expressed as the mean ± S.E. (*error bars*), *n* = 4. *, *p < 0.05; **, *p < 0.01.
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sequent delivery to the plasma membrane whereupon CD8+ T cells recognize non-self peptide-loaded MHC I molecules on the surface of the cell via the CD8+ T cell receptor, leading to activation of T cell antiviral effector functions.

Our data are consistent with previous studies demonstrating that a reduction of TAP1 leads to a dramatic reduction of peptide transport into the ER and a reduction of antigen loading and MHC class I antigen presentation, as TAP1 abundance is predictive of the TAP-mediated antigen transport into the ER (41, 42). The data presented in this paper also suggest a somewhat unconventional model whereby TAP1 ubiquitination and degradation do not result in a similar change in TAP2 abundance. However, previous studies have demonstrated that newly synthesized TAP2 requires TAP1 for appropriate folding and stability (43). Moreover, TAP2 is targeted for proteasomal degradation when it is expressed in the absence of TAP1 in T2 lymphoblastoid cells or TAP1 abundance is reduced in vitro (43). Conversely, introduction of TAP1 into TAP1-deficient melanoma cells markedly enhances TAP2 protein levels (44). By contrast, our experiments suggest that Cif may target TAP1 for degradation after it has associated with, and promoted the folding of, TAP2. It is likely that the stability of the TAP heterodimer differs after biosynthesis, when interactions with chaperone proteins may alter the protein half-life. For example, tapasin is a chaperone protein that provides protein stability to the TAP complex, and TAP2 is known to interact with tapasin independently of TAP1 (45–47). We envision a scenario where tapasin-TAP2 interactions maintain TAP2 stability even when TAP1 levels are reduced by Cif. Further studies are needed to examine the protein stability of TAP2 and the mechanism by which TAP2 abundance is maintained in the presence of Cif and TAP1 degradation.

This study is the first to demonstrate that a bacterial virulence factor inhibits TAP abundance and function. Because disrupting TAP and class I MHC function significantly disrupts CD8+ T cell responses to virus infection, it is not surprising that many viruses have evolved a variety of strategies to subvert or evade host T cell responses, with TAP and MHC class I molecules being frequent targets (48, 49). For example, ICP47 produced by herpes simplex virus-1 and -2, and US6, produced by the human cytomegalovirus, inhibit TAP function by disrupting peptide binding and translocation into the ER, respectively (50–55). Epstein-Barr virus inhibits TAP function and antigen presentation by utilizing two proteins, LMP-1 and BCRF1, to disturb the balance between TAP1 and TAP2 expression, thereby reducing functional TAP heterodimer abundance (56–58). Bovine herpesvirus-1 encodes a protein, UL49.5, that acts in several varicellosviruses to inhibit TAP by promoting the ubiquitination and proteasomal degradation of TAP. In this system, degradation of TAP results in a down-regulation of MHC class I molecules at the cell surface (59–61). The present study identifies the first bacterial, or nonviral protein, that promotes TAP degradation and significantly reduces peptide translocation into the ER and inhibits MHC class I antigen presentation at the cell surface of human airway epithelial cells.

We recently published a study showing that Cif, by inhibiting USP10 activity, also promotes the ubiquitination and degradation of the CFTR chloride channel in airway epithelial cells, which will decrease mucociliary clearance of respiratory pathogens (27). Thus, taken together with the present study, our data suggest that Cif is acting at two levels to promote polymicrobial infections in the airways of patients colonized with P. aeruginosa. First, Cif promotes the degradation of the CFTR chloride channel, a key component in regulating the volume of airway surface liquid, and thereby reduces mucociliary clearance of pathogens leading to a chronic P. aeruginosa infection. Second, Cif also inhibits MHC class I-mediated viral and bacterial antigen presentation, primarily by reducing TAP1 abundance and antigen transport into the ER. This effect of Cif will decrease viral and bacterial clearance from the lungs. Accordingly, we suggest these combined actions of the P. aeruginosa Cif virulence factor promote the establishment of polymicrobial infections in the lungs of patients with cystic fibrosis, chronic obstructive pulmonary disease, and bronchiectasis and provide a novel explanation for the clinical observation that patients infected with P. aeruginosa who acquire a viral infection have a higher viral load and a longer duration of viral infection than patients who are not simultaneously infected with P. aeruginosa (5, 62–65).

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