The *Pseudomonas aeruginosa* LasB Metalloproteinase Regulates the Human Urokinase-Type Plasminogen Activator Receptor through Domain-Specific Endoproteolysis[▽]

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Pseudomonas aeruginosa is an opportunistic pathogen in human lungs, where its secretable LasB metalloproteinase can be a virulence factor. The urokinase-type plasminogen activator receptor (uPAR) participates in pericellular proteolysis and the adherence/migration of epithelial cells and leukocytes recruited during infection and shows functional regulation by various proteinases via limited endoproteolysis occurring within its three domains (D1 to D3). We thus examined the proteolytic activity of LasB on uPAR by using recombinant uPAR as well as uPAR-expressing, human monocytic, and bronchial epithelial cell lines. Protein immunoblotting and flow immunocytometry using a panel of domain-specific anti-uPAR antibodies showed that LasB is able to cleave uPAR both within the sequence linking D1 to D2 and at the carboxy terminus of D3. Comparison of LasB-producing and LasB-deficient bacterial strains indicated that LasB is entirely responsible for the uPAR cleavage ability of *P. aeruginosa*. Based on amino-terminal protein microsequencing and mass spectrometry analysis of the cleavage of peptides mimicking the uPAR sequences targeted by LasB, cleavage sites were determined to be Ala⁸⁴-Val⁸⁵ and Thr⁸⁶-Tyr⁸⁷ (D1-D2) and Gln²⁷⁹-Tyr²⁸⁰ (D3). Such a dual cleavage of uPAR led to the removal of amino-terminal D1, the generation of a truncated D2D3 species, and the shedding of D2D3 from cells. This proteolytic processing of uPAR was found to (i) drastically reduce the capacity of cells to bind urokinase and (ii) abrogate the interaction between uPAR and the matrix adhesive protein vitronectin. The LasB proteinase is thus endowed with a high potential for the alteration of uPAR expression and functioning on inflammatory cells during infections by *P. aeruginosa*.

Pseudomonas aeruginosa is a gram-negative bacterium that is a major opportunistic pathogen in humans and is capable of infecting various tissues and organs and causing severe, damaging, and often fatal pathologies (42). The lung is a main target for colonization and infection by the bacteria either in the context of a chronic, progressively deteriorating infectious and inflammatory pulmonary disease such as cystic fibrosis (CF) or in a more acute setting such as severe pneumonia in immunocompromised patients (28, 45, 55). A number of secretable bacterial virulence factors can contribute to the pathogenicity of *P. aeruginosa*, including proteinases (32, 42, 45). Among the pseudomonal proteinases is an elastolytic metalloproteinase of the thermolysin/M4 peptidase family that is encoded by the *lasB* gene, hereafter designated LasB but also known as pseudolysin or *P. aeruginosa* elastase (36, 38, 41). LasB, a type II secretion system substrate, has received much attention because of its highly regulated expression during tissue colonization and infection, particularly in the lungs (55), and its potential as a virulence factor (32, 41, 45). Indeed, *P. aeruginosa*-associated pathogenic processes include both damage to tissues via hydrolysis of extracellular matrix (ECM)

components and alterations of endothelial and epithelial barriers (3, 15, 36, 41). It also participates in the proteolytic inactivation of numerous extracellular components of the innate and adaptive immune systems including opsonins, cytokines, chemokines, antibacterial peptides, and proteinase inhibitors (1, 9, 35, 46, 51). In addition, more recent studies have shown that the exposure of isolated human leukocytes to LasB results in an inhibition of cell chemotaxis and phagocytotic and/or microbicidal activities via proteolytic alterations of membrane immune receptors (9, 24, 36).

The urokinase-type plasminogen activator (uPA) receptor (uPAR, also designated CD87 or Mo3 antigen) is a highly glycosylated glycosylphosphatidylinositol (GPI)-anchored cell membrane protein. It is composed of three homologous globular domains, named D1, D2, and D3, from the amino terminus to the carboxy terminus of the molecule, each containing a conserved arrangement of disulfide bonds and separated by short interdomain linker sequences (43). uPAR is ubiquitously expressed in human tissues, being present on the surface of many leukocyte subsets as well as on endothelial and epithelial cells (8, 12, 53). Its expression is increased upon the exposure of cells to a wide range of inflammatory mediators (12, 53). uPAR is a pleiotropic receptor that is pivotal in (patho)physiological processes involving cell migration and tissue remodeling, particularly during infection and inflammation. Thus, uPAR binds with high affinity to the serine (Ser) proteinase uPA, which, once bound, catalyzes the conversion of plasminogen into plasmin. The latter activates several matrix metallo-

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proteinases (MMPs), thus conferring a high potential for pericellular proteolysis, ECM processing, and cell motility to cells expressing uPAR (8, 43). uPAR also participates in cell adherence and migration in other ways, including (i) its capacity to bind the ECM adhesive protein vitronectin (Vn) (44), (ii) a physical and functional interaction with various integrins (30), and (iii) an intrinsic chemotactic activity that is associated with a peptide motif present within the interdomain sequence linking domains D1 and D2 and which targets various types of inflammatory cells (39). In accordance with these properties, uPAR-deficient mice show an impaired recruitment and activation of leukocytes at sites of infection, resulting in impaired bacterial clearance and increased host mortality (19, 39), as demonstrated in a model of *P. aeruginosa* pulmonary infection (20). The use of uPAR-deficient animal models also indicates that uPAR may play a prominent role in tissue repair and healing after an inflammatory event (59).

uPAR is highly susceptible to endoproteolysis within the D1-D2 linker sequence and/or at the carboxy terminus of D3. These two regions contain cleavage sites for proteinases involved in the inflammatory response, such as uPA, plasmin, and various MMPs, as well as Ser proteinases secreted from activated leukocytes (4, 5, 23, 29). Since the D1 domain plays a crucial role in the interactions of uPAR with its various ligands, the endoproteolytic removal of D1 is thus a likely pathway for controlling pericellular proteolysis as well as cell adherence and migration (8, 40, 44). Indeed, soluble uPAR species (suPAR), which include the free D1 domain but also truncated D2D3 forms as well as full-length (D1D2D3) species, can be detected in the biological fluids of healthy individuals (40). Importantly, suPAR concentrations are markedly increased in infectious and inflammatory disorders and can be of prognostic value for a negative outcome, including in lung diseases (40).

Despite the prominent role played by uPAR in the host's capacity to mount adequate innate and adaptive immune responses to pathogens, particularly in the lungs (20, 39), to date, only a few studies have investigated a direct interaction between bacteria or bacterial secreted products and uPAR (6, 13, 26). In this context, the present study provides new insights into the proteolytic regulation of leukocytic and epithelial uPAR by demonstrating that the major *P. aeruginosa* proteinase, LasB, generates membrane and/or soluble truncated forms of this receptor and thus has an impact on the interactions between uPAR and its physiological ligands.

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MATERIALS AND METHODS

Reagents. Recombinant human uPAR, corresponding to the sequence Leu¹-Arg²⁸¹ and fused to a carboxy-terminal polyhistidine tag (rhuPAR-His₆), was obtained from R&D Systems (Minneapolis, MN). Fluorescein isothiocyanate (FITC)-conjugated high-molecular-weight human uPA was obtained from American Diagnostica (Greenwich, CT), and bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) (5,100 U/mg) was obtained from Sigma-Aldrich (Saint-Louis, MO). Purified human plasma Vn was obtained from BioSource (Camarillo, CA). Purified LasB [EC 3.4.24.26; referred to as M04.005 in subclan MA(E) in the MEROPS peptidase database (<http://merops.sanger.ac.uk>)] isolated from a pathogenic strain of *P. aeruginosa* was obtained from Elastin Products Company (Owensville, MS). It was stored at -80°C as a 0.5-mg/ml stock

solution ($\approx 15\ \mu\text{M}$, based on a molecular mass of 33 kDa) and had a specific activity of 260 units/mg protein. The fungal thermolysin/LasB-specific inhibitor *N*-(α -rhamnopyranosyloxyhydroxyphosphinyl)-Leu-Trp (phosphoramidon [PA]) (27) and the LasB substrate elastin-Congo red were obtained from Sigma. The following two synthetic N-acylated and C-amidated peptides mapping the human uPAR sequences Ser⁸¹-Cys⁹⁵ within the D1-D2 linker domain and Asn²⁷²-Gly²⁸³ at the carboxy terminus of D3, respectively (43), were prepared with $>95\%$ purity by Eurogentec (Seraing, Belgium); the 15-mer peptide SGRAVYTSRSR YLEC, hereafter designated the D1-D2 peptide, and the 12-mer peptide NHP DLDVQYRSG, hereafter designated the D3 peptide.

Antibodies used in this study were anti-uPAR mouse monoclonal antibodies (mAbs) 3931 (against the D1 domain) and 3932 (against the D2 domain) (both immunoglobulin G1 [IgG1] mAbs) and 3936, an IgG2a mAb reacting with an undetermined epitope in the D2D3 portion of human uPAR (4) (all from American Diagnostica); mouse IgG1 mAbs R2 and R4 directed against different epitopes located in the D3 domain (23, 33) and provided by Gunilla Høyer-Hansen (The Finsen Laboratory, Copenhagen, Denmark); mouse IgG2b mAb MY4 from Coulter Corp. (Miami, FL); mouse mAbs ICRF44 (IgG1) and IB4 (IgG2a), directed against epitopes on the CD11b/ α_M and CD18/ β_2 subunits of the $\alpha_M\beta_2$ integrin, respectively, as well as nonspecific control mAbs of the IgG1, IgG2a, or IgG2b subclass from Ancell (Bayport, MN); His probe (H-15), a rabbit polyclonal antibody against the His₆ peptide tag, from Santa Cruz Biotechnologies (Santa Cruz, CA); a rabbit polyclonal antibody directed against the mature, 33-kDa LasB protein obtained from a commercial source; an FITC-conjugated F(ab')₂ goat antibody against mouse IgG from Dako (Glostrup, Denmark); and horseradish peroxidase (HRP)-conjugated antibodies against mouse IgG (ImmunoPure) from Pierce (Rockford, IL) or from Jackson Laboratories (West Grove, PA) (as were HRP-conjugated anti-rabbit IgG goat antibodies [AffiniPure]).

Cell culture reagents were all obtained from Gibco BRL (Paysley, Scotland), except for heat-inactivated fetal calf serum, which was obtained from HyClone (Logan, UT), whereas chemicals for cell solubilization, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and protein electrotransfer were obtained from Sigma or from Bio-Rad (Hercules, CA).

Cell cultures and membrane protein biotinylation. (i) Monocytic cells. The U937 human promonocytic cell line (50) (ATCC CRL-1593.2; American Type Culture Collection, Manassas, VA) was grown and differentiated into a monocyte/macrophage phenotype by exposure to phorbol 13-myristate 12-acetate (Sigma-Aldrich) (4). Differentiated adherent U937 cells were recovered, washed, and, when needed, surface biotinylated using sulfo-succinimidyl-6-(biotinamido)hexanoate (EZ-Link Sulfo-NHS-LC-biotin; Pierce, Rockford, IL) as previously reported (4). Cells were finally resuspended in Hanks' balanced salt solution (HBSS) at 1×10^6 cells/ml for further treatment (see below).

(ii) Respiratory epithelial cells. 16HBE14o⁻ cells, hereafter designated 16HBE cells, are human simian virus 40-transfected bronchial epithelial cells (14) and were a gift from D. C. Gruenert (University of Vermont, Colchester, VT). Cells were cultured to confluence in 24-well plates (Techno Plastic Products, Trasadingen, Switzerland) as described previously (17), and when required, biotinylation of cell surface molecules was performed using EZ-Link Sulfo-NHS-LC-biotin (5). Cell monolayers were washed twice with HBSS before further treatment (see below).

(iii) *P. aeruginosa* CM. The *P. aeruginosa* PAO1 reference strain producing LasB protease and strain PAO1*lasB9Δ* (referred to as PDO240), in which the *lasB* gene sequence had been deleted and replaced with a spectinomycin resistance DNA fragment (37), were grown overnight at 37°C in Luria broth (LB) up to stationary phase. Bacterium-free culture supernatants were obtained by a double-step centrifugation, including a first run at $6,000 \times g$ for 10 min followed by a second one at $12,000 \times g$ for 10 min. Supernatants were further filtered through 0.45- μm membranes to provide conditioned media (CM), hereafter designated PAO1-CM and PDO240-CM, respectively, which were immediately frozen in aliquots at -80°C when used for uPAR cleavage experiments (see below). A subfraction was submitted to tetra-acetic acid precipitation for protein content analysis (see below). The elastolytic activity present in PAO1-CM and in PDO240-CM was measured using elastin-Congo red as a substrate and increasing concentrations of purified LasB in the range of 12.5 to 100 nM for calibration, exactly as previously described (10) except that the incubation time for various dilutions of CM or of LasB with the substrate was 30 min.

Exposure of recombinant uPAR- or uPAR-expressing cells to LasB or to *P. aeruginosa* CM. (i) rhuPAR. rhuPAR-His₆ adjusted to 1 $\mu\text{g/ml}$ (20 nM) in 50 mM Tris-100 mM NaCl (pH 7.4) was mixed either with purified LasB in the range of 15 to 300 nM (final concentrations; enzyme-to-substrate [E/S] molar ratio of $\sim 1:1$ to $15:1$) or with a preformed mixture of LasB and 50 μM PA or was left without proteinase as a control, and incubation then proceeded for 5 to 30 min

at 37°C. In some experiments, rhuPAR-His₆ was incubated in parallel with increasing dilutions of PAO1-CM or of PDO240-CM for 30 min or, as control, with the LB culture medium alone or diluted CM preincubated with 50 μ M PA. At the end of the exposure of rhuPAR-His₆ to the protease or to media, the enzymatic activity was neutralized by the addition of PA, and samples were immediately solubilized in the presence of 2% (wt/vol) SDS and 5 mM *N*-ethylmaleimide (SDS-NEM) by boiling at 100°C for 5 min in the presence of 5% (vol/vol) 2-mercaptoethanol for the reduction of disulfide bonds when required. In some experiments, part of the samples was kept unsolubilized and stored at -20°C until the interaction of rhuPAR-His₆ with immobilized Vn was measured (see below).

(ii) U937 cell suspensions. Suspensions of U937 cells in HBSS received 1 mM of both CaCl₂ and MgCl₂ (HBSS-CaMg), LasB in the range of 37.5 to 150 nM was then added, and incubation continued at 37°C from 5 to 40 min before the enzyme was blocked with 50 μ M PA. In some experiments, part of the suspensions was incubated in parallel with 0.5 U/ml PI-PLC. A fraction of each cell sample was kept at 4°C for fluorescence-activated cell sorting (FACS) analysis (see below). The remaining cells were pelleted by centrifugation at 530 $\times$ *g* for 15 min at 4°C and then solubilized as described below, whereas extracellular fluids were collected and further centrifuged at 18,000 $\times$ *g* for 30 min at 4°C to eliminate cell debris before being directly solubilized with SDS-NEM, as described above, or stored at -80°C.

(iii) 16HBE cell monolayers. 16HBE cell monolayers covered with 200 μ l HBSS-CaMg received various amounts of LasB in the range of 37.5 to 600 nM, and incubation continued at 37°C from 15 to 60 min before the enzymatic activity was blocked with 50 μ M PA. In parallel incubations, cell monolayers were exposed to 1 U/ml PI-PLC. Extracellular fluids were collected and kept as described above. 16HBE cell monolayers were washed twice with HBSS and then solubilized as described below.

SDS-PAGE and immunoblot analysis. **(i) Cell solubilization and isolation of biotinylated proteins.** U937 cells in suspension (2 $\times$ 10⁷ cells/ml) and 16HBE cell monolayers were solubilized in the presence of a cocktail of protease inhibitors, the concentration of extracted soluble proteins was measured, and biotinylated membrane proteins were separated from nonbiotinylated proteins and finally solubilized in SDS-NEM exactly as previously described (4, 5).

(ii) SDS-PAGE and immunoblotting. rhuPAR-His₆ (10 to 15 ng per lane), biotinylated cell membrane proteins (extracted from 5 μ g of total U937 or 16HBE cell proteins per lane), or proteins present in the extracellular fluids (30 μ l per lane, corresponding to the equivalent of 2.5 or 15 μ g of total cell proteins for U937 or 16HBE cells, respectively) were separated by SDS-PAGE, as previously described (4, 5), with either intact or reduced disulfide bonds. Following SDS-PAGE, proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA), and uPAR-related protein species were probed by immunoblotting, as previously described (4, 5), by using a panel of domain-specific, anti-uPAR antibodies as detailed in Results and in the figure legends. The chemiluminescent reaction kit ECL+ (Amersham Biosciences, Little Chalfont, United Kingdom) was used to reveal immune complexes in immunoblotting analysis. Calibration of gels for measurement of relative molecular weight (*M_r*) was done using Broad Range or Kaleidoscope prestained standard proteins (Bio-Rad).

For analysis of protein content in *P. aeruginosa* culture CM, tetra-acetic acid-precipitated protein samples were suspended in SDS sample buffer and loaded onto a 12% polyacrylamide gel for SDS-PAGE. Proteins in polyacrylamide gels were either stained with Coomassie blue or transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) together with the Kaleidoscope prestained standard proteins. Immunoblot detection of the LasB protein was performed using a primary anti-LasB rabbit antibody at a 1:500 dilution and a HRP-conjugated anti-rabbit IgG goat antibody at a dilution of 1:5,000 in Tris-buffered saline containing 10% (wt/vol) skim milk and 0.1% (vol/vol) Tween 20. The presence of the protein was revealed using the Supersignal Pico chemiluminescence revelation kit (Pierce).

Determination of cleavage sites for LasB within uPAR. **(i) Fragmentation of D1-D2 and D3 peptides by LasB and mass spectrometry analysis.** Peptides reconstituted at 2 mg/ml in sterile deionized water were adjusted to 65 μ M in HBSS and incubated at 37°C in a 10- μ l volume with LasB (E/S ratio, 1:25 or 1:100) for 5 or 15 min. Controls consisted of proteinase-free peptides or of proteinase dilutions alone incubated at 37°C for the longest periods of time in each given experiment. Enzymatic activity was stopped by acidifying the reaction mixture. Samples were immediately lyophilized before being processed for mass analysis using matrix-assisted laser desorption/ionization-time of flight mass spectrometry on a Voyager-DE STR spectrometer (Applied Biosystems Inc., Framingham, MA) exactly as previously described (4). Identification of the truncated peptides was carried out with GPMW software, version 6.0 (Light-

house Data, Odense, Denmark), for the D1-D2 and D3 sequences. Searching was performed using monoisotopic masses with a mass tolerance of 0.1%.

(ii) Amino-terminal amino acid sequencing. Protein carrier-free rhuPAR-His₆ was resuspended in HBSS at 100 μ g/ml (2 μ M) and was incubated overnight at 37°C with 10 or 100 nM of LasB (E/S ratio, 1:20 or 1:200) or left untreated as a control. Proteins were then solubilized and reduced as described above before SDS-PAGE separation and transfer onto polyvinylidene difluoride membranes. Protein bands corresponding to the D2D3 species (see Results for details) were visualized by amido black staining, excised, and subjected to five cycles of amino-terminal microsequencing using an Applied Biosystems ABI 494 protein sequencer.

FACS analysis. Following exposure to LasB, U937 cells were distributed at 10⁵ cells per well in conical-bottom 96-well microplates (Nunc, Roskilde, Denmark). After centrifugation, cells were immunolabeled for 1 h at 4°C in 0.1 ml of HBSS-CaMg containing 0.25% (wt/vol) bovine serum albumin (BSA) (Euromedex, Strasbourg, France) (HBSS-CaMg-BSA) and the primary mAb at 5 μ g/ml, washed, and then incubated with a secondary FITC-conjugated goat anti-mouse F(ab')₂ antibody (4). The one-step FITC-labeled uPA binding assay was performed as detailed previously (4). Briefly, cells pelleted in microplates were resuspended in 0.1 ml of HBSS-CaMg-BSA containing 50 nM of either FITC-uPA or FITC-F(ab')₂ as a control fluoresceinated protein and then incubated for 1 h at 4°C before being washed three times. Quantitative binding of FITC-labeled proteins to the surface of the selected viable cells (defined as those remaining negative for labeling with propidium iodide, performed immediately before fluorescence measurement) was measured using a FACScan cytometer coupled to CellQuest 3.3 software (Becton Dickinson, Franklin Lakes, NJ) and expressed as the geometric mean of fluorescence intensity histograms after background binding provided by nonspecific IgG isotypes or FITC-F(ab')₂ had been subtracted.

Measurement of soluble extracellular uPAR. The concentration of suPAR in cell-free U937 or 16HBE extracellular fluids was determined by use of a specific quantitative enzyme-linked immunosorbent assay (ELISA) (Quantikine ELISA for human uPAR; R&D Systems) according to the manufacturer's instructions. The detection limit of the assay was 62.5 pg/ml suPAR.

Vn-uPAR interaction assay. The capacity of LasB-treated rhuPAR-His₆ to bind Vn was assayed essentially according to a previously reported procedure (47). Briefly, Maxisorb 96-well plates (Nunc) were coated with 5 μ g/ml purified Vn in a phosphate-buffered saline (PBS) solution (0.1 ml/well) overnight at 4°C and then saturated with 1% BSA in PBS (PBS-BSA) (0.2 ml/well) for 1 h at room temperature. Wells then received 5 nM uPA together with rhuPAR-His₆ (final concentration, 10 nM), which had been previously exposed to either LasB in the range of 30 to 150 nM for 30 min at 37°C, before LasB was blocked with 50 μ M PA, or 150 nM LasB premixed with 50 μ M PA or left unexposed to the proteinase. All reactants were diluted in PBS-BSA (0.1 ml/well), and incubation continued for 1 h at 4°C. After washing in PBS containing 0.1% (vol/vol) Tween 20, wells were probed for bound rhuPAR-His₆ using anti-D3 mAb R2 or R4 (2 μ g/ml), followed by a HRP-conjugated rabbit anti-mouse Ig antibody (1/1,000 dilution). After washing, the immune complexes were quantified through a colorimetric assay for HRP activity using the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (KPL, Gaithersburg, MD). Following acidification of the medium, the optical density (OD) in wells was measured at 405 nm in an ELISA plate reader. Vn-bound uPAR was calculated by subtracting the background OD measured in wells that did not receive rhuPAR-His₆ and was expressed as the percentage of the binding measured with rhuPAR-His₆ that was not treated with LasB.

Statistical analysis. Results are expressed as means $\pm$ standard errors of the means (SEM) for the indicated number of experiments. Statistical analysis was performed by use of the univariate Student's *t* test for paired samples implemented using StatView software, version 5.0 (Abacus Concepts, Berkeley, CA).

RESULTS

Soluble uPAR is cleaved by LasB within both the D1-D2 linker and D3 carboxy-terminal sequences. In order to assess the direct proteolytic activity of LasB on the human urokinase receptor, the recombinant protein rhuPAR-His₆ was exposed to the proteinase, and the molecular species generated were analyzed by immunoblotting following SDS-PAGE. The initial full-length, three-domain (D1D2D3) rhuPAR-His₆, with a molecular mass of ~64 kDa (mean value over four determina-

FIG. 1. Cleavage of the D1-D2 linker and of the D3 carboxy-terminal sequences within soluble uPAR by LasB. rhuPAR-His₆ (1 μ g/ml, ~20 nM) was either exposed to purified LasB in the range of 15 to 150 nM or left untreated as a control (lanes labeled uPAR) for 30 min at 37°C. Proteins (15 ng rhuPAR-His₆ per lane) were then separated by SDS-PAGE on 12% polyacrylamide gels under reducing conditions and immunoblotted using anti-D2 mAb 3932 at 0.05 μ g/ml (middle), anti-D1 mAb 3931 at 1 μ g/ml (left), or anti-His₆ His probe polyclonal antibody at 0.1 μ g/ml to label the carboxy-terminal polyhistidine tag (right). Bound antibodies were detected by an appropriate HRP-coupled secondary antibody followed by chemiluminescent detection of HRP activity. Portions of films corresponding to the location of the relevant antigens and representative of at least three different analyses are illustrated. Indicated on the right are the positions and molecular masses (in kDa) of standard proteins used for electrophoretic calibration, whereas arrows on the left indicate the positions of the full-length, three-domain rhuPAR-His₆ (D1D2D3) and of the truncated species (D2D3 and free D1); note that the initial uPAR protein unexposed to LasB contains minute amounts of D2D3 and free D1 (lanes labeled uPAR in middle and left panels, respectively).

tions), was found, as expected, to react with both mAb 3931 (anti-D1) and mAb 3932 (anti-D2) (Fig. 1, left and middle panels, respectively) as well as with mAb R2 (anti-D3) (data not shown). However, when exposed to LasB in the range of 15 to 150 nM for 30 min and then immunoblotted with mAb 3932, rhuPAR-His₆ appeared to be converted, in a LasB concentration-dependent manner, into smaller molecular species of ~43 kDa. These fragments did not react with the anti-D1 mAb 3931 (Fig. 1, compare left and middle panels) but were still reactive with mAb R2 (data not shown), thus corresponding to a truncated receptor composed of the D2 and D3 domains (D2D3) (4, 23). The conversion of rhuPAR-His₆ into D2D3 was already detectable 5 min after exposure to 75 nM LasB and was complete at 30 min (data not shown and Fig. 1, middle, respectively). It should be noted that minute amounts of a D2D3-like species were present in the rhuPAR-His₆ sample before exposure to LasB (Fig. 1, middle). These were accompanied by traces of the free D1 domain with a mass of ~18.5 kDa, as judged by immunoblotting with mAb 3931 (Fig. 1, left). Interestingly, the efficient LasB-induced conversion of rhuPAR-His₆ into the D2D3 species, which resisted further endoproteolysis up to 150 nM LasB (Fig. 1, middle), was not accompanied by an accumulation of free D1 in the reaction mixture. Indeed, this species was undetectable at the highest proteinase concentrations (Fig. 1, left), and no D1-derived subfragments could be detected by immunoblotting. Similar results were observed when intramolecular disulfide bonds were maintained for SDS-PAGE (data not shown). Altogether, these data demonstrated that LasB can efficiently cleave soluble uPAR within the sequence linking D1 to D2, while the released D1 domain is likely to be extensively degraded.

Some proteinases that are active on the D1-D2 linker sequence of uPAR have been shown to also target the carboxy terminus of the receptor (4, 5). To evaluate whether LasB

could do so, proteinase-treated rhuPAR-His₆ was assayed by immunoblotting for the presence of the carboxy-terminal polyhistidine epitope. The initial three-domain rhuPAR-His₆ produced signals of similar intensities with both the anti-D2 mAb 3932 and the anti-His₆ antibody (Fig. 1, middle and right panels, respectively). The truncated D2D3 species resulting from the cleavage of D1 by LasB also showed a similar reactivity with the two probes, up to 75 nM LasB. However, higher concentrations (i.e., 150 nM) resulted in a drastic reduction in the reactivity of D2D3 with the anti-His₆ antibody (Fig. 1, compare middle and right panels), thus indicating that the bacterial proteinase is indeed able to cleave uPAR at its carboxy terminus.

Cleavage sites in the D1-D2 linker sequence were identified through amino-terminal sequencing of the truncated D2D3 species resulting from exposure of rhuPAR-His₆ to LasB. These D2D3 molecules were found to contain a predominant species with the amino-terminal sequence YSRSR, while at the lowest E/S ratio (1:200), a less abundant species with an amino-terminal VTYSR sequence could also be detected (<10% of the total D2D3 material analyzed on an amino acid molar basis). These results thus established that cleavage sites for LasB in the amino-terminal part of uPAR are at the Ala⁸⁴-Val⁸⁵ and at the Thr⁸⁶-Tyr⁸⁷ peptide bonds (43). Because this approach was not applicable to the characterization of the cleavage sites at the carboxy terminus of D3, these were determined through matrix-assisted laser desorption/ionization-time of flight mass spectrometry analysis of the proteolytic fragmentation of a synthetic peptide, which mimicked the D3 carboxy-terminal sequence ahead of the last cysteine residue engaged in intradomain disulfide bonding (43). Exposure of this peptide (sequence COO-NHPDLVDVQYRSG-NH₂) (monoisotopic ion [M + H]⁺ value, 1,441.6466) to LasB for 5 to 15 min and at E/S ratios of 1:100 to 1:25 resulted in a marked but nevertheless incomplete hydrolysis, producing one single shorter detectable species with an [M + H]⁺ value of 979.4548 (values are representative of two determinations). This corresponded to peptide COO-NHPDLVDVQ-NH₂, thus demonstrating a single cleavage at the Gln-Tyr peptide bond, analogous to Gln²⁷⁹-Tyr²⁸⁰ in human uPAR (43). A similar approach applied to a synthetic peptide mimicking the D1-D2 linker sequence (COO-SGRAVTYSR-YLEC-NH₂) confirmed the capacity of LasB to specifically cleave the Ala-Val and Thr-Tyr peptide bonds (data not shown).

LasB is the principal protease secreted by *P. aeruginosa* that can target uPAR. The secretory proteome (secretome) of clinical strains of *P. aeruginosa* is known to contain a number of proteases other than LasB, including the elastolytic metalloprotease LasA and the Ser proteinase PrpL (or protease IV) (10, 49). In order to establish whether or not LasB was uniquely responsible for the cleavage of uPAR, we compared the proteolytic capacity of the secretome from the LasB-producing strain PAO1 to that of the LasB-deficient strain PDO240, designed on the PAO1 genetic background (37). One-dimensional SDS-PAGE analysis of proteins present in the bacterial culture CM coupled to specific immunoblotting confirmed that LasB is a major product in the secretome of strain PAO1, as previously reported (49), whereas it was undetectable in the secretome of strain PDO240 (Fig. 2A). Measurement of the proteolytic activity present in the CM, measured on elastin-Congo red, confirmed that PAO1-CM

FIG. 2. LasB is a major product in the secretome of *P. aeruginosa* and the principal proteinase for cleavage of uPAR. (A) Proteins secreted into the culture medium at the stationary growth phase of a LasB-producing (PAO1) or of a LasB-deficient (PDO240) strain of *P. aeruginosa* were precipitated and separated by SDS-PAGE in 12% polyacrylamide gels. This was followed by staining with Coomassie blue (top) or transfer onto nitrocellulose membranes for immunoblot (IB) detection of LasB protein (bottom) using an anti-LasB antibody at a 1:500 dilution and a HRP-conjugated secondary antibody followed by chemiluminescent detection. The asterisk indicates the position of the LasB protein with a mass of ~ 33 kDa, which was missing in PDO240-CM. (B) rhuPAR-His₆ (1 μ g/ml) was either left untreated as a reference (lane labeled uPAR) or exposed to purified LasB in the range of 12.5 to 100 nM or to various dilutions (1/5 to 1/40) of PAO1-CM or of PDO240-CM for 30 min at 37°C. Controls included LasB (100 nM) or culture medium (1/5 dilutions) preincubated with the LasB inhibitor PA (50 μ M) before the addition of rhuPAR-His₆ or incubation in LB culture medium alone (lane labeled +LB). Proteins were then separated by SDS-PAGE and immunoblotted (IB) using anti-D2 mAb 3932 (top) and then anti-D1 mAb 3931 (bottom) exactly as described in the legend to Fig. 1. Portions of films corresponding to the location of the relevant antigens and representative of two different experiments are shown.

contained a marked activity, which was found to be approximately equivalent to that produced by 500 nM purified LasB (mean value of three determinations) and was inhibited to a mean of 85% by the LasB-specific inhibitor PA (27) and to 90% by 75 mM EDTA, as previously reported (10, 17). Conversely, barely detectable elastolytic activity was observed in PDO240-CM ($\leq 5\%$ of that measured in PAO1-CM), in agreement with previous observations (37). When exposed to these milieus and then assayed for immunoblotting with anti-D2 mAb 3932, rhuPAR-His₆ was found to be readily cleaved by PAO1-CM and progressively converted into the truncated D2D3 species for dilutions of the milieu ranging from 1/5 to 1/40, i.e., equivalent to 100 to 12.5 nM LasB. The pattern of cleavage was similar to that resulting from the exposure of rhuPAR-His₆ to purified LasB in the same range of concentrations (Fig. 2B, top). Importantly, cleavage was totally blocked by the preincubation of PAO1-CM with the inhibitor PA, whereas it was undetectable when rhuPAR-His₆ was exposed to PDO240-CM at the lowest dilution (1/5), compared to the control incubations made in LB culture medium alone or in the presence of PA (Fig. 2B, top). Moreover, immunoblotting with anti-D1 mAb 3931 showed that the freed D1 domain was degraded at the highest levels of protease activities (Fig. 2B, bottom).

LasB cleaves the D1-D2 linker domain from membrane uPAR on human monocytic and respiratory epithelial cells. Within the airways, the resident intra-alveolar population of monocytes/macrophages and the epithelial cells composing the respiratory mucosal lining are two major cell types that constitutively express uPAR on their surface and increase its expression upon an inflammatory stimulation (12, 53). Both populations are thus likely targets for a potential activity of LasB on cell-associated uPAR. This possibility was explored by using two human cell lines known to express uPAR, the promonocytic cell line U937, differentiated into macrophage-like cells by exposure to the phorbol ester phorbol

13-myristate 12-acetate (4, 53), and the bronchial epithelial cell line 16HBE (5).

The proteolytic activity of LasB on membrane uPAR was first explored on U937 cells through flow immunocytometry. Exposure of cells to the proteinase in the range of 30 to 300 nM for 30 min resulted in a LasB concentration-dependent decrease in binding of anti-D1 mAb 3931 to a maximal reduction of epitope surface expression of $\sim 85\%$ (Fig. 3A). By contrast, the binding of mAbs 3932 and 3936, both targeting D2D3, did not significantly decrease following cell exposure to LasB up to 300 nM. Time course analyses indicated that the binding of mAb 3931 was already reduced by $\sim 20\%$ and $\sim 50\%$ after 10 and 20 min of exposure to 75 nM LasB, respectively. Incubation of cells with LasB premixed with its inhibitor PA maintained the binding of mAb 3931 to levels observed in control cells, indicating that the impact of LasB on the expression of the D1 domain relied on the proteinase activity. No change in the binding capacity of U937 cells exposed to LasB could be noted when antibodies directed towards cell surface antigens such as CD11b, CD18, and CD14 were used, thus indicating a rather restricted activity of LasB on leukocytic membrane proteins (data not shown). Finally, the exposure of U937 cells to up to 300 nM LasB did not increase the number of propidium iodide-positive dead cells, indicating that LasB was not cytotoxic under our experimental conditions (data not shown).

We next used immunoblotting analysis coupled to surface protein biotinylation and extraction and SDS-PAGE to evaluate the impact of LasB on membrane uPAR (4, 5). As previously observed by us and by others (4, 23, 53), the bulk of membrane uPAR in U937 cells migrated as a full-length, three-domain (D1D2D3) species with a molecular mass in the range of 60 to 80 kDa. This provided a strong signal when probed with anti-D1 mAb 3931 (Fig. 3B, left). However, a minor truncated two-domain (D2D3) species with a molecular mass in the range of 45 to 60 kDa was also initially present on

FIG. 3. Generation of a truncated membrane uPAR species on monocytic and respiratory epithelial cells exposed to LasB. Cells were surface biotinylated and then exposed to purified LasB or incubated in the absence of proteinase (not treated [NT]) at 37°C before blockade of the activity of the proteinase, separation of cells from extracellular milieu, and analysis of the structure of membrane uPAR. (A) Membrane uPAR domain expression on monocytic U937 cells exposed to increasing LasB concentrations for 30 min was analyzed by FACS using a panel of mAbs (5 μ g IgG/ml) directed at different epitopes within uPAR, as indicated. Data are expressed as the percentage of the mean fluorescence intensity (MFI) value obtained for control cells incubated in the absence of proteinase, as described in Materials and Methods, and are illustrated as means $\pm$ SEM of values obtained in eight independent experiments. MFI values (means $\pm$ SEM) measured for each mAb on control cells were 288 ± 103 for anti-D1 mAb 3931, 113 ± 23 for anti-D2 mAb 3932, and $1,059 \pm 217$ for anti-D2D3 mAb 3936. (B) Biotinylated membrane proteins extracted from 5 μ g of total proteins solubilized from LasB-treated or nontreated U937 cells were separated by SDS-PAGE on 10% polyacrylamide gels under reducing conditions. Immunoblotting (IB) was then performed with mAb 3931 (anti-D1, 0.25 μ g/ml) (left) or mAb 3932 (anti-D2, 0.05 μ g/ml) (right), as described in the legend to Fig. 1. One representative experiment out of three independent ones is illustrated. Brackets on the left indicate the positions of the D1D2D3 and D2D3 membrane uPAR species, identified by their range of M_r s as well as differential reactivities with the mAbs. (C) Biotinylated membrane proteins extracted from 5 μ g of total proteins solubilized from LasB-treated or nontreated epithelial 16HBE cell monolayers (150 nM LasB for 15 to 60 min [left] or 150 to 600 nM LasB for 30 min [right]) were separated by SDS-PAGE on 15% acrylamide gels and immunoblotted under reducing conditions with mAb 3932 (0.5 μ g/ml). The figure illustrates one representative experiment out of three independent ones.

the surface of U937 cells not exposed to LasB, as detected by anti-D2 mAb 3932 (Fig. 3B, compare right and left panels, respectively). The smear-like appearance of uPAR in macrophage-differentiated U937 cells is known to be due to high levels of heterogeneous glycosylation (53). Immunoblotting analysis of membrane uPAR extracted from U937 cells exposed for 30 min to LasB ranging from 30 to 150 nM then confirmed that the bacterial proteinase could induce the cleavage of the D1 domain in cell surface-associated uPAR in a concentration-dependent manner. Indeed, the exposure of cells to LasB drastically reduced signals observed at the position of D1D2D3 species (Fig. 3B, left), while it increased labeling at the position of the truncated D2D3 species (Fig. 3B, right), compared to control cells.

Similar effects of LasB on membrane uPAR were observed when 16HBE bronchial epithelial cell monolayers were exposed to the proteinase. As for the monocytic cells, SDS-PAGE/immunoblotting analysis of biotinylated 16HBE cell membrane proteins indicated that epithelial membrane uPAR occurred as two molecular species (5) (here well focused in SDS-polyacrylamide gels), i.e., the major D1D2D3 form with a molecular mass of ~ 72 kDa (mean value over five determinations) and a minor truncated D2D3 species of ~ 53 kDa (Fig. 3C). Exposure of cells to 150 nM LasB resulted in a marked conversion of full-length uPAR into the D2D3 species as early

as 15 min, which was almost complete by 60 min (Fig. 3C, left). Increasing the concentration of LasB up to 600 nM over a 30-min period did not result in more extensive proteolysis of the truncated membrane uPAR (Fig. 3C, right).

LasB induces shedding of membrane uPAR. Besides the removal of domain D1 from membrane-associated uPAR as described above, LasB also resulted in the shedding of soluble forms of the truncated receptor. Indeed, immunoblotting analysis of cell-free extracellular milieu using anti-D2 mAb 3932 revealed that the exposure of 16HBE epithelial cell monolayers to LasB in the range of 150 to 600 nM and from 15 to 60 min led to an increasing release of a soluble species showing a molecular mass of ~ 45 kDa (Fig. 4A), which was unreactive with anti-D1 mAb 3931 (data not shown), thus corresponding to the truncated D2D3 form. The identity of this species was confirmed by comparison with those resulting from the exposure of cells to a bacterial PI-PLC. This phospholipase hydrolyzes the GPI structure between the inositol phosphate ring and the diacylglycerol moiety (43) and thus released full-length (molecular mass, ~ 61 kDa) and truncated (molecular mass, ~ 47 kDa) uPAR species in a ratio that mirrored that observed for membrane uPAR on normal 16HBE cells (compare Fig. 4A and 3C). Immunoblot analysis of the milieu taken from monocytic U937 cell suspensions exposed to LasB (Fig. 4B) similarly showed that part of the membrane D2D3 species

FIG. 4. Shedding of soluble species of uPAR from respiratory epithelial and monocytic cells by LasB. U937 cell suspensions or 16HBE cell monolayers were exposed at 37°C to milieu without (not treated [NT]) or with LasB under conditions of enzyme concentration and duration of exposure similar to those described in the legend to Fig. 3 or to 0.5 U/ml PI-PLC (+PI-PLC) for 30 min. (A and B) Extracellular milieus were separated from cells and solubilized with SDS, and soluble proteins released from a number of 16HBE cells (A) or U937 cells (B), corresponding to 15 μ g or 2.5 μ g of total cell proteins, respectively, were submitted to SDS-PAGE under reducing conditions and then immunoblotted with anti-D2 mAb as described in the legend to Fig. 3. A and B illustrate one representative experiment out of five (16HBE cells) or three (U937 cells) independent ones. (C) Measurement by ELISA of soluble uPAR present in the extracellular fluids of 16HBE cell monolayers exposed for 30 min to control milieu (NT), to LasB at the indicated concentrations, or to 0.5 U/ml PI-PLC (+PI-PLC) (means $\pm$ SEM of values obtained in four independent experiments). *, $P \leq 0.06$ (versus nontreated samples).

generated by the bacterial proteinase was shed into the extracellular environment for enzyme concentrations of ≥ 75 nM. It is important to note that free D1 domains were never detected in the extracellular milieu of LasB-treated cells using our immunoblotting approach, thus suggesting that this species was efficiently degraded by the proteinase, as has been noted with recombinant uPAR.

Use of an ELISA specific for human suPAR allowed a more quantitative evaluation of receptor shedding. Indeed, suPAR was significantly increased above its basal value when 16HBE cells were exposed to LasB in the range 75 to 300 nM to reach maximal levels that were similar to those observed with PI-PLC-treated cells (Fig. 4C). Similar quantification performed on U937 cell-derived milieu indicated that 150 nM LasB released 6.0 ± 2.2 ng/ml suPAR from the monocytic cells, compared to 3.8 ± 1.4 ng/ml and 27.2 ± 5.0 ng/ml ($n = 3$) measured in the extracellular milieu of control untreated and PI-PLC-treated cells, respectively.

Interaction of uPAR with uPA and Vn is abrogated following cleavage of the receptor by LasB. Membrane uPAR is a binding site for two ligands with major importance in pericellular proteolysis and cell adherence, i.e., the proteinase uPA and the adhesive protein Vn (8, 44). Amino-terminal domains of both ligands can interact with several discrete sequences located within all three domains of uPAR, although the presence of domain D1 is mandatory for high-affinity interactions of the ligands with the receptor (43, 44). Since LasB can proteolyti-

cally remove domain D1, we used an FITC-coupled uPA binding assay to ascertain that the proteinase can affect the interaction of uPA with the U937 cell surface. Exposure to LasB for 30 min indeed resulted in an enzyme concentration-dependent reduction of the cell capacity to bind FITC-uPA. This was closely related to the specific disappearance of domain D1 as judged through the decreased binding of anti-D1 mAb 3931, as opposed to the unchanged binding of anti-D2 mAb 3932 (Fig. 5A). Binding of uPA-FITC reached a maximal 65% reduction at the highest LasB concentration tested, i.e., 150 nM. As previously observed (4, 43), the exposure of U937 cells to 0.5 U/ml PI-PLC resulted in a similar decrease in the binding capacity of uPA (64% and 69% reduction by two determinations) via the partial shedding of full-length uPAR, as shown in Fig. 4.

We then assayed the capacity of soluble uPAR to bind to Vn immobilized on microplates through an ELISA-type procedure using anti-D3 mAbs to probe and quantitate bound uPAR (47). As previously reported (44, 47), the interaction of uPAR with Vn was found to require the prior occupancy of uPAR by uPA (data not shown). Exposure of rhuPAR-His₆ to increasing concentrations of LasB in the range of 30 to 150 nM prior to the assay was observed to progressively impair the binding of uPAR to Vn (Fig. 5B, top). This effect was concomitant with cleavage within the D1-D2 linker sequence and the transition from full-length uPAR to the D2D3 species, as evaluated by immunoblotting with anti-D2 mAb 3932 (Fig. 5B, bottom).

FIG. 5. Alteration of the capacity of U937 cells exposed to purified LasB to bind uPA and impaired capacity of LasB-treated uPAR to bind to Vn. (A) U937 cells were exposed to LasB ranging from 30 to 150 nM or incubated under control conditions as described in the legend to Fig. 3. Cells were then incubated for 1 h at 4°C with 50 nM FITC-uPA or with mAbs 3931 (anti-D1) or 3932 (anti-D2), both at 5 µg/ml, and binding was assessed by FACS analysis. Data were expressed as a percentage of the MFI value obtained for control cells incubated in the absence of proteinase and are illustrated as means $\pm$ SEM of values obtained in three independent experiments. Specific MFI values (means $\pm$ SEM) measured for each ligand on control cells were 135 ± 17 for anti-D2 3932 mAb, 137 ± 45 for anti-D1 3931 mAb, and 101 ± 41 for FITC-uPA. *, $P < 0.07$ (versus untreated cell samples). (B) rhuPAR-His₆ was either left untreated (NT) or exposed to LasB in the range of 30 to 150 nM for 30 min at 37°C before the enzymatic activity was blocked with PA or to 150 nM LasB premixed with 50 µM PA (150/PA). Vn-coated wells (5 µg/ml) were then incubated for 1 h at 4°C with reaction mixtures containing 10 nM rhuPAR-His₆ in the presence of 5 nM uPA. Bound uPAR was revealed by sequential incubations with anti-D3 mAb R2 or R4, a HRP-conjugated anti-mouse IgG antibody, and the chromogenic substrate 3,3',5,5'-tetramethylbenzidine before the OD at 405 nm was measured. Vn-bound rhuPAR-His₆ (top) was expressed as the percentage of the OD value measured with intact, nontreated rhuPAR-His₆ after subtraction of the background OD measured in rhuPAR-His₆-free wells. In parallel, cleavage of rhuPAR-His₆ by LasB (bottom) was monitored by immunoblotting using anti-D2 mAb 3932 as described in the legend to Fig. 1. The figure shows data obtained in one representative experiment out of five.

Exposure of rhuPAR-His₆ to LasB premixed with its inhibitor PA left the soluble receptor uncleaved and maintained its full interaction with immobilized Vn (Fig. 5B).

DISCUSSION

It is well established that many pathogenic bacterial species use their own and/or some of the host proteolytic systems to serve several purposes during infection, including escape from host defenses through the proteolysis of extracellular as well as cell-associated immune effector proteins (31, 34, 38, 56). Proteinases secreted by *P. aeruginosa* are exemplary in this respect, and even though not all clinical isolates of *P. aeruginosa* produce LasB (11), for those that do, this particular proteinase may be an important candidate for providing the microorganism with immunoevasive capacities (9, 17, 24).

We have now established that the human uPAR is a target for LasB, which is able to efficiently cleave discrete peptide bonds in the D1-D2 linker sequence and to generate a truncated (D2D3) species. The exposure of soluble or cell-associated uPAR to the proteinase in the range of 15 to 75 nM and for periods of time as short as 5 to 15 min already results in a

FIG. 6. Proteolytic processing of human uPAR by the *P. aeruginosa* LasB metalloproteinase. Cleavage sites for LasB within the D1-D2 linker sequence (Asn⁷⁷-Glu⁹⁴) and at the carboxy terminus of domain D3 (Asn²⁷²-Gly²⁸³) are indicated by arrows, as assumed from mass spectrometry analysis of the fragmentation of a synthetic peptide mimicking the D3 carboxy terminus (filled arrowhead) by LasB and from amino acid amino-terminal microsequencing of the D2D3 species generated from recombinant human uPAR exposed to the enzyme (open arrowheads). The minimal active sequence (SRSRY) of the chemotactic epitope embedded in the D1-D2 linker sequence is underlined.

marked proteolysis of D1. As a bacterial Zn²⁺-dependent metalloproteinase of the thermolysin family, LasB cleaves at the amino group side of the amino acid located at the P'1 position in the sessile peptide bond, preferably a bulky apolar or hydrophobic amino acid, whereas the presence of Ala at the P1 position increases the rate of hydrolysis (38, 41). The cleavage sites identified within D1-D2, Ala⁸⁴-Val⁸⁵ and Thr⁸⁶-Tyr⁸⁷ (Fig. 6), are in agreement with the enzymatic properties of LasB, and indeed, hydrolysis at Xaa-Val and Xaa-Tyr peptide bonds has previously been reported for this proteinase (1, 17, 41). Interestingly, a truncated (D2D3) species corresponding to cleavage at the Thr⁸⁶-Tyr⁸⁷ position was found to be the most abundant species following the interaction of uPAR with LasB, and this position is also a major cleavage site identified within the D1-D2 linker region for some mammalian metalloproteinases of the MMP family known to shed domain D1 and to regulate uPAR-dependent cell functions (2, 29). Because soluble free D1 remains undetectable by our immunoblot assay, we propose that the removal of D1 by LasB is accompanied by a further, extensive degradation of this domain. A similar feature has been previously observed for some proteinases that are active on uPAR (2, 4, 5), indicating a marked susceptibility of this important functional domain to endoproteolysis.

However, the activity of LasB on uPAR is not limited to the D1-D2 linker sequence, as the proteinase could also shed a soluble form of the receptor from the surface of both leukocytic and epithelial cells. This likely occurs through cleavage at a single peptide bond within the carboxy terminus of domain D3, as indicated by (i) the disappearance of the polyhistidine tag placed carboxy terminally in our recombinant soluble protein (Fig. 1) and (ii) the cleavage by LasB of a synthetic peptide mimicking the D3 carboxy-terminal sequence Asn²⁷²-Gly²⁸³ at a position corresponding to the Gln²⁷⁹-Tyr²⁸⁰ peptide bond in human uPAR (43) (Fig. 6). Because of the highly efficient proteolysis of the D1 domain by LasB, suPAR appeared in extracellular milieus only as the truncated D2D3 species, which could be detected with LasB concentrations as low as 75 nM (Fig. 4). At the highest concentrations tested in our studies (i.e., ≥ 150 nM) and after 30 min, LasB was able to release a quantity of suPAR from the surface of respiratory epithelial cells equivalent to that shed by a bacterial phospholipase acting on the GPI moiety. In contrast, when applied to monocytic cells, LasB was markedly less efficient for shedding uPAR than was PI-PLC, which is known to release about 50% of membrane-associated uPAR (4), while LasB released about 5%. This infers that the accessibility of LasB to the juxtamembrane

region of uPAR may vary depending on the cell type and/or membrane environment in which this receptor is expressed. In contrast to free D1, the D2D3 species generated by LasB resisted further degradation by the proteinase and accumulated on the cell surface and in the extracellular milieu, even at the highest LasB concentration used in our study (i.e., 600 nM). LasB is thus a new member, and the first of bacterial origin, of a group of proteinases, also including the polymorphonuclear leukocyte Ser proteinases neutrophil elastase and cathepsin G as well as plasmin, which are capable of cleaving both the D1-D2 linker and the D3 carboxy terminus of human uPAR and of releasing soluble D2D3 species (4, 5). In this respect, it remains to be determined whether LasB can generate soluble D2D3 molecules endowed with chemotactic and promigratory activities. Indeed, we show here that it cleaves the D1-D2 linker domain slightly upstream of the S⁸⁸RSRY sequence, which has been identified as being the minimal chemotactic epitope (8, 39, 40) (Fig. 6).

Experimental and/or clinical evidence points to a role for LasB as a major modulator of host cellular responses during *P. aeruginosa* infections. As mentioned previously, LasB is able to alter the activity of many immune and inflammatory human cells, including leukocytes and epithelial cells, by proteolytically acting on membrane receptors (9, 17, 24, 56). It is noteworthy that the concentrations of LasB (in the range of 10 to 100 nM) as well as the duration of exposure to the proteinase (within 60 min) usually found to be effective for the cleavage of domains of different receptors are similar to those reported here for uPAR and appear to be lower than those required for the cleavage of extracellular immune effector proteins (1, 35, 51). LasB quantitatively represents one of the major proteins/proteases secreted in culture milieu by various strains of *P. aeruginosa*, including clinical isolates from CF patients (49) (Fig. 2A), and is the only one that targets uPAR, as established in the present study by showing that the secretome from a LasB-deficient strain does not produce cleavage of the receptor, in contrast to that of a LasB-producing strain. Furthermore, the mucopurulent respiratory liquid from such patients has been found to markedly stimulate the transcription of the *lasB* gene (57). Indeed, both transcripts of *lasB* (51) and the LasB protein (25, 54) can be detected in sputum obtained from infected CF patients. Specific quantitative immunodetection or measurement of the enzymatic activity indicates that LasB concentrations in sputum or bronchoalveolar lavage fluid are highly variable between patients, ranging from a few nmol/liter up to levels of ≥ 300 nM (25, 54). Because the protein material experimentally extracted from sputum as well as lavage fluid represents diluted airway fluids, one could thus expect the local concentrations of LasB in the immediate vicinity of host cells to be higher than the concentrations measured ex vivo and to be well within the range of those active on uPAR. Finally, it has been shown that high titers of antibodies are continuously produced against LasB in many chronically infected CF patients, suggesting a sustained secretion of the proteinase from the time of initial infection (16), while respiratory fluids obtained from such patients contain host protein degradation products, some of which may be specific markers for LasB activity in vivo (1, 9, 51). Taken together, these observations provide strong support for the assumption that LasB, among the numerous *P. aeruginosa* proteinases, may be an important

virulence factor (7, 16), particularly by dampening the host immune responses through the alteration of membrane receptors. This may well apply to uPAR, since it has been shown that experimental *P. aeruginosa* corneal infections in mice are accompanied by fragmentation of the receptor, leading to the shedding of D1 and the appearance of truncated D2D3 species in the infected tissues (6). Along with this experimental observation, there is recent clinical evidence that suPAR measured in sputum from patients with various infectious/inflammatory lung diseases is found at the highest levels in a series of CF patients all infected with *P. aeruginosa*, with an approximately 10-fold increase in concentrations compared to those found in healthy subjects (58). Although the proteolytic system(s) responsible for the shedding of uPAR in these in vivo settings has not yet been characterized, our present in vitro demonstration that LasB can target uPAR suggests that it may well be a major participant in the proteolytic processing of the receptor, together with host proteolytic systems such as the leukocyte Ser proteinases and the uPA/plasmin/MMP cascade (4, 5, 23, 29).

We have confirmed in this study that the proteolysis of D1, as it results from LasB activity on uPAR, has a major negative impact on the capacity of cells to bind uPA. Such a cleavage of uPAR may constitute a survival advantage for the bacteria at various levels. First, the engagement of uPAR through its D1 domain with uPA and with membrane partners of the integrin family is a major molecular mechanism involved in recruitment and in bactericidal activities of inflammatory leukocytes that are triggered in response to various pathogens, including *P. aeruginosa* (19, 20, 39). The disruption of these molecular interactions due to the removal of D1 may participate, together with the proteolytic alteration of other immune effectors (1, 9, 32), in the inappropriate dampening of the early immune response seen during *P. aeruginosa* infections and may thus help the bacteria to escape rapid clearance and to colonize the respiratory tract (28, 55). Subsequently, as for many pathogenic bacteria (31), *P. aeruginosa* appears to be able to use the host uPA/plasmin/MMP cascade, besides its own proteinases, to facilitate its dissemination in tissues through matrixolysis. This occurs through the binding of plasminogen on the bacterial surface and its activation by uPA (18) as well as through a bypass of the uPAR/uPA-dependent controlled activation of MMPs via the removal of D1 and the direct unbalanced activation of MMPs by LasB (15). Moreover, more unusual interactions between *P. aeruginosa* and uPA appear to exist, since in vitro, uPA was able to markedly increase bacterial growth under stringent culture conditions (low oxygen and low bacterial density) and to stimulate the production of bacterial proteinases (21, 22). By decreasing the uPAR-dependent uptake of uPA by host cells, cleavage of uPAR may thus increase uPA bioavailability to *P. aeruginosa*, with the consequent stimulation of both bacterial growth and dissemination.

Finally, cleavage of uPAR may well participate in the detrimental progression of lung tissues chronically infected with *P. aeruginosa* towards fibrosis, bronchiectasis, and emphysema (28, 52, 55). Indeed, the proteolysis of D1 by LasB expectedly abrogates the capacity of uPAR to bind, in a uPA-dependent manner, the inflammatory ECM component Vn (8, 44). The trimolecular interaction between uPAR, uPA, and Vn supports the migration and adherence of airway monocytes/macrophages to the Vn-rich provisional ECM and, subsequently,

its remodeling and elimination during tissue healing (48), whereas the proliferation and migration of smooth muscle and epithelial cells within inflammatory ECM also depend partly on the engagement of uPAR with uPA and Vn and with membrane integrins (8, 44). A disruption of these molecular axes through of removal of D1 may thus have profound negative impacts on tissue repair and the return to homeostasis (59).

Although the biological consequences of the interactions between the *P. aeruginosa* proteinase LasB and the host receptor uPAR require further investigation, we hypothesize that the cleavage and shedding of uPAR could represent an important step during the complex interplay between the host and the pathogen, allowing the latter to more easily infect and colonize susceptible tissues, such as those of the respiratory tract.

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REFERENCES

- Alcorn, J. F., and J. R. Wright. 2004. Degradation of pulmonary surfactant protein D by *Pseudomonas aeruginosa* elastase abrogates innate immune function. *J. Biol. Chem.* **279**:30871–30879.
- Andolfo, A., W. R. English, M. Resnati, G. Murphy, F. Blasi, and N. Sidenius. 2002. Metalloproteases cleave the urokinase-type plasminogen activator receptor in the D1-D2 linker region and expose epitopes not present in the intact soluble receptor. *Thromb. Haemost.* **88**:298–306.
- Azghani, A. O. 1996. *Pseudomonas aeruginosa* and epithelial permeability: role of virulence factors elastase and exotoxin A. *Am. J. Respir. Cell Mol. Biol.* **15**:132–140.
- Beaufort, N., D. Leduc, J.-C. Rousselle, V. Magdolen, T. Luther, A. Namane, M. Chignard, and D. Pidard. 2004. Proteolytic regulation of the urokinase receptor/CD87 on monocyte cells by neutrophil elastase and cathepsin G. *J. Immunol.* **172**:540–549.
- Beaufort, N., D. Leduc, J.-C. Rousselle, A. Namane, M. Chignard, and D. Pidard. 2004. Plasmin cleaves the juxtamembrane domain and releases truncated species of the urokinase receptor (CD87) from human bronchial epithelial cells. *FEBS Lett.* **574**:89–94.
- Berk, R. S., M. Katar, Z. Dong, and D. E. Day. 2001. Plasminogen activators and inhibitors in the corneas of mice infected with *Pseudomonas aeruginosa*. *Investig. Ophthalmol. Vis. Sci.* **42**:1561–1567.
- Blackwood, L. L., R. M. Stone, B. H. Iglewski, and J. E. Pennington. 1983. Evaluation of *Pseudomonas aeruginosa* exotoxin A and elastase as virulence factors in acute lung infection. *Infect. Immun.* **39**:198–201.
- Blasi, F., and P. Carmeliet. 2002. uPAR: a versatile signalling orchestrator. *Nat. Rev. Mol. Cell Biol.* **3**:932–943.
- Buret, A., and A. W. Cripps. 1993. The immunoevasive activities of *Pseudomonas aeruginosa*. Relevance for cystic fibrosis. *Am. Rev. Respir. Dis.* **148**:793–805.
- Caballero, A. R., J. M. Moreau, L. S. Engel, M. E. Marquart, J. M. Hill, and R. J. O'Callaghan. 2001. *Pseudomonas aeruginosa* protease IV enzyme assays and comparison to other *Pseudomonas* proteases. *Anal. Biochem.* **290**:330–337.
- Cabrol, S., A. Olliver, G. B. Pier, A. Andreumont, and R. Ruimy. 2003. Transcription of quorum-sensing system genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. *J. Bacteriol.* **185**:7222–7230.
- Chu, E. K., J. Cheng, J. S. Foley, B. H. Mecham, C. A. Owen, K. J. Haley, T. J. Mariani, I. S. Kohane, D. J. Tschumperlin, and J. M. Drazen. 2006. Induction of the plasminogen activator system by mechanical stimulation of human bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **35**:628–638.
- Coleman, J. L., and J. L. Benach. 2003. The urokinase receptor can be induced by *Borrelia burgdorferi* through receptors of the innate immune system. *Infect. Immun.* **71**:5556–5564.
- Cozens, A. L., M. J. Yezzi, K. Kunzelmann, T. Ohnui, L. Chin, K. Eng, W. E. Finkbeiner, J. H. Widdicombe, and D. C. Gruenert. 1994. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **10**:38–47.
- de Bentzmann, S., M. Polette, J.-M. Zahm, J. Hinnrasky, C. Kileztky, O. Bajolet, J.-M. Klossek, A. Filloux, A. Lazdunski, and E. Puchelle. 2000. *Pseudomonas aeruginosa* virulence factors delay airway epithelial wound repair by altering the actin cytoskeleton and inducing overactivation of epithelial matrix metalloproteinase-2. *Lab. Invest.* **80**:209–219.
- Döring, G., W. Goldstein, A. Röhl, P. O. Schiötz, N. Høiby, and K. Botzenhart. 1985. Role of *Pseudomonas aeruginosa* exoenzymes in lung infections of patients with cystic fibrosis. *Infect. Immun.* **49**:557–562.
- Dulon, S., D. Leduc, G. S. Cottrell, J. D'Alayer, K. K. Hansen, N. W. Bunnett, M. D. Hollenberg, D. Pidard, and M. Chignard. 2005. *Pseudomonas aeruginosa* elastase disables proteinase-activated receptor 2 in respiratory epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **32**:411–419.
- Ferreira da Silva, C. M., L. de Abreu Vidipo, R. Nishi, and M. C. Plotkowski. 2004. Binding of plasminogen to *Pseudomonas aeruginosa* results in formation of surface-associated plasmin and enhanced bacterial invasiveness. *Microb. Pathog.* **36**:59–66.
- Gyetko, M. R., D. Aizenberg, and L. Mayo-Bond. 2004. Urokinase-deficient and urokinase receptor-deficient mice have impaired neutrophil antimicrobial activation in vitro. *J. Leukoc. Biol.* **76**:648–656.
- Gyetko, M. R., S. Sud, T. Kendall, J. A. Fuller, M. W. Newstead, and T. J. Standiford. 2000. Urokinase receptor-deficient mice have impaired neutrophil recruitment in response to pulmonary *Pseudomonas aeruginosa* infection. *J. Immunol.* **165**:1513–1519.
- Hart, D. A., and D. E. Woods. 1994. Human urokinase, a serine proteinase, potentiates the in-vitro growth of micro-organisms which commonly infect burn patients. *J. Med. Microbiol.* **41**:264–271.
- Heys, S. J. D., S. L. Orton, and D. G. Allison. 1999. Effect of urokinase on the extracellular virulence properties of *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *J. Basic Microbiol.* **39**:17–24.
- Høyer-Hansen, G., U. Pessara, A. Holm, J. Pass, U. Weidle, K. Danø, and N. Behrendt. 2001. Urokinase-catalysed cleavage of the urokinase receptor requires an intact glycolipid anchor. *Biochem. J.* **358**:673–679.
- Ijiri, Y., K. Matsumoto, R. Kamata, N. Nishino, R. Okamura, T. Kambara, and T. Yamamoto. 1994. Suppression of polymorphonuclear leucocyte chemotaxis by *Pseudomonas aeruginosa* elastase in vitro: a study of the mechanisms and the correlation with ring abscess in pseudomonal keratitis. *Int. J. Exp. Pathol.* **75**:441–451.
- Jaffar-Bandjee, M.-C., A. Ladzunski, M. Bally, J. Carrère, J.-P. Chalazette, and C. Galabert. 1995. Production of elastase, exotoxin A, and alkaline protease in sputa during pulmonary exacerbation of cystic fibrosis in patients chronically infected by *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* **33**:924–929.
- Jin, H., Y. P. Song, G. Boel, J. Kochar, and V. Pancholi. 2005. Group A streptococcal surface GAPDH, SDH, recognizes uPAR/CD87 as its receptor on the human pharyngeal cell and mediates bacterial adherence to host cells. *J. Mol. Biol.* **350**:27–41.
- Kessler, E., M. Safrin, W. R. Abrams, J. Rosenbloom, and D. E. Ohman. 1997. Inhibitors and specificity of *Pseudomonas aeruginosa* LasA. *J. Biol. Chem.* **272**:9884–9889.
- Knapp, S., M. J. Schultz, and T. van der Poll. 2005. Pneumonia models and innate immunity to respiratory bacterial pathogens. *Shock* **24**(Suppl. 1):12–18.
- Koolwijk, P., N. Sidenius, E. Peters, C. F. M. Sier, R. Hanemaaijer, F. Blasi, and V. W. M. van Hinsbergh. 2001. Proteolysis of the urokinase-type plasminogen activator receptor by metalloprotease-12: implication for angiogenesis in fibrin matrices. *Blood* **97**:3123–3131.
- Kugler, M. C., Y. Wei, and H. A. Chapman. 2003. Urokinase receptor and integrin interactions. *Curr. Pharmaceut. Design* **9**:1565–1574.
- Lähtenmäki, K., S. Edelman, and T. K. Korhonen. 2005. Bacterial metastasis: the host plasminogen system in bacterial invasion. *Trends Microbiol.* **13**:79–85.
- Lau, G. W., D. J. Hassett, and B. E. Britigan. 2005. Modulation of lung epithelial functions by *Pseudomonas aeruginosa*. *Trends Microbiol.* **13**:389–397.
- List, K., G. Høyer-Hansen, E. Rønne, K. Danø, and N. Behrendt. 1999. Different mechanisms are involved in the antibody mediated inhibition of ligand binding to the urokinase receptor: a study based on biosensor technology. *J. Immunol. Methods* **222**:125–133.
- Maeda, H., and T. Yamamoto. 1996. Pathogenic mechanisms induced by microbial proteases in microbial infections. *Biol. Chem. Hoppe-Seyler* **377**:217–226.
- Matheson, N. R., J. Potempa, and J. Travis. 2006. Interaction of a novel form of *Pseudomonas aeruginosa* protease (aeruginolysin) with interleukin-6 and interleukin-8. *Biol. Chem.* **387**:911–915.
- Matsumoto, K. 2004. Role of bacterial proteases in pseudomonal and serratal keratitis. *Biol. Chem.* **385**:1007–1016.
- Melver, K. S., E. Kessler, J. C. Olson, and D. E. Ohman. 1995. The elastase propeptide functions as an intramolecular chaperone required for elastase

- activity and secretion in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **18**:887–889.
38. Miyoshi, S., and S. Shinoda. 2000. Microbial metalloproteases and pathogenesis. *Microbes Infect.* **2**:91–98.
 39. Mondino, A., and F. Blasi. 2004. uPA and uPAR in fibrinolysis, immunity and pathology. *Trends Immunol.* **25**:450–455.
 40. Montuori, N., V. Visconte, G. Rossi, and P. Ragno. 2005. Soluble and cleaved forms of the urokinase-receptor: degradation products or active molecules? *Thromb. Haemost.* **93**:192–198.
 41. Morihara, K., and J. Y. Homma. 1985. *Pseudomonas* proteases, p. 41–79. In I. A. Holder (ed.), *Bacterial enzymes and virulence*. CRC Press, Boca Raton, FL.
 42. Pier, G. B., and R. Ramphal. 2005. *Pseudomonas aeruginosa*, p. 2587–2615. In G. L. Mandel, J. E. Bennet, and R. Dolin (ed.), *Mandell, Douglas and Bennet's principles and practice of infectious diseases*, 6th ed. Churchill Livingstone, New York, NY.
 43. Ploug, M. 2003. Structure-function relationships in the interaction between the urokinase-type plasminogen activator and its receptor. *Curr. Pharmaceut. Design* **9**:1499–1528.
 44. Preissner, K. T., S. M. Kanse, and A. E. May. 2000. Urokinase receptor: a molecular organizer in cellular communication. *Curr. Opin. Cell Biol.* **12**: 621–628.
 45. Sadikot, R. T., T. S. Blackwell, J. W. Christman, and A. S. Prince. 2005. Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. *Am. J. Respir. Crit. Care Med.* **171**:1209–1223.
 46. Schmidtchen, A., I.-M. Frick, E. Andersson, H. Tapper, and L. Björck. 2002. Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Mol. Microbiol.* **46**:157–168.
 47. Sidenius, N., A. Andolfo, R. Fesce, and F. Blasi. 2002. Urokinase regulates vitronectin binding by controlling urokinase receptor oligomerization. *J. Biol. Chem.* **277**:27982–27990.
 48. Simon, D. I., N. K. Rao, H. Xu, Y. Wei, O. Majdic, E. Rønne, L. Kobzik, and H. A. Chapman. 1996. Mac-1 (CD11b/CD18) and the urokinase receptor (CD87) form a functional unit on monocytic cells. *Blood* **88**:3185–3194.
 49. Sriramulu, D. D., M. Nimtz, and U. Romling. 2005. Proteome analysis reveals adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis lung environment. *Proteomics* **5**:3712–3721.
 50. Sundstrom, C., and K. Nilsson. 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int. J. Cancer* **17**:565–577.
 51. Suter, S. 1994. The role of bacterial proteases in the pathogenesis of cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **150**:S118–S122.
 52. Taggart, C. C., C. M. Greene, T. P. Carroll, S. J. O'Neill, and N. G. McElvaney. 2005. Elastolytic proteases. Inflammation resolution and dysregulation in chronic infective lung disease. *Am. J. Respir. Crit. Care Med.* **171**:1070–1076.
 53. Todd, R. F., I. F. Mizukami, S. D. Vinjamuri, R. D. Trochelman, W. W. Hancock, and D. Y. Liu. 1990. Human mononuclear phagocyte activation antigens. *Blood Cells* **16**:167–182.
 54. Tournier, J.-M., J. Jacquot, E. Puchelle, and J. G. Bieth. 1985. Evidence that *Pseudomonas aeruginosa* elastase does not inactivate the bronchial inhibitor in the presence of leukocyte elastase. Studies with cystic fibrosis sputum and with pure proteins. *Am. Rev. Respir. Dis.* **132**:524–528.
 55. Tümmler, B., and C. Kiewitz. 1999. Cystic fibrosis: an inherited susceptibility to bacterial respiratory infections. *Mol. Med. Today* **5**:351–358.
 56. Vollmer, P., I. Walev, S. Rose-John, and S. Bhakdi. 1996. Novel pathogenic mechanism of microbial metalloproteinases: liberation of membrane-anchored molecules in biologically active form exemplified by studies with the human interleukin-6 receptor. *Infect. Immun.* **64**:3646–3651.
 57. Wolfgang, M. C., J. Jyot, A. L. Goodman, R. Ramphal, and S. Lory. 2004. *Pseudomonas aeruginosa* regulates flagellin expression as part of a global response to airway fluid from cystic fibrosis patients. *Proc. Natl. Acad. Sci. USA* **101**:6664–6668.
 58. Xiao, W., Y.-P. Hsu, A. Ishizaka, T. Kirikae, and R. B. Moss. 2005. Sputum cathelicidin, urokinase plasminogen activation system components, and cytokines discriminate cystic fibrosis, COPD, and asthma inflammation. *Chest* **128**:2316–2326.
 59. Zhang, G., H. Kim, X. Cai, J. M. Lopez-Guisa, C. E. Alpers, Y. Liu, P. Carmeliet, and A. A. Eddy. 2003. Urokinase receptor deficiency accelerates renal fibrosis in obstructive nephropathy. *J. Am. Soc. Nephrol.* **14**:1254–1271.

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