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*J Immunol* 2004; 172:540-549; doi: 10.4049/jimmunol.172.1.540

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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Proteolytic Regulation of the Urokinase Receptor/CD87 on Monocytic Cells by Neutrophil Elastase and Cathepsin G

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The urokinase receptor (CD87) participates to the pericellular proteolytic potential of migrating cells and to the recruitment of leukocytes during inflammation. It consists of three structurally homologous domains, with the C-terminal domain D3 attached to cell membranes through a GPI anchor. CD87 is susceptible to an endoproteolytic processing removing the N-terminal domain D1 and generating truncated D2D3 membrane species, thus modulating CD87-associated functions. Full-length or truncated CD87 can be also released from cells via juxtacell membrane cleavage by phospholipases and/or by yet unidentified proteases. Using a recombinant CD87 and the CD87-positive monocytic U937 cell line and isolated blood monocytes, we show by protein immunoblotting and flow immunocytometry that the human neutrophil serine-proteases elastase and cathepsin G cleave CD87 within the D1-D2 linker sequence, while in addition cathepsin G is highly efficient in cleaving the C-terminus of D3. The combination of cathepsin G and elastase provided by degranulated neutrophils results in enzymatic cooperation leading to the release from monocytic cells of a truncated D2D3 species resembling that previously described in pathological body fluids. Using mass spectrometry analysis, the proteolytic fragmentation of synthetic peptides mapping the D1-D2 linker and D3 C-terminal domains identifies potential cleavage sites for each enzyme and suggests the existence of a mechanism regulating the CD87(D1-D2)-associated chemotactic activity. Finally, isolated or combined elastase and cathepsin G drastically reduce the capacity of cells to bind urokinase. Secretable leukocyte serine-proteases are thus endowed with high potential for the regulation of CD87 expression and function on inflammatory cells. The Journal of Immunology, 2004, 172: 540–549.

It is also expressed to high levels in many types of tumor cells and strongly contributes to their invasiveness and metastatic potential (4, 5). Indeed, CD87 is a multiligand receptor that operates as a key element in (patho)physiological processes involving cell migration and tissue remodeling, particularly during inflammation and cancer metastasis. Thus, CD87 binds with high affinity the Ser-protease urokinase-type plasminogen activator (uPA) which, once bound, catalyzes the conversion of plasminogen into plasmin, the latter participating in turn to the activation of various matrix metalloproteinases (MMPs), thus conferring to cells expressing CD87 a high potential for pericellular proteolysis (2, 4, 6). CD87 also binds the extracellular matrix adhesive protein vitronectin, thus greatly contributing to the adherence of leukocytic cells (7). High-affinity binding of both uPA and vitronectin to CD87 requires the presence of D1 (2, 7). Yet, CD87 participates in cell adherence and migration through two other routes: 1) a physical association and functional interaction with various integrins, including the leukocyte α5β1/CD11b-CD18 (8–10), and 2) an intrinsic chemotactic activity associated to the D1-D2 linker sequence (11). In accordance with these properties demonstrated in vitro, CD87-deficient mice show an impaired recruitment of leukocytes in response to local infections, resulting in increased mortality (12–14).

CD87 is highly susceptible to endoproteolysis within the D1-D2 linker sequence, which contains cleavage sites for proteases involved in the inflammatory response, such as uPA itself, plasmin, and various MMPs (2, 15–19), and endoproteolytic processing of CD87 with removal of D1 is a likely pathway for controlling pericellular proteolysis as well as cell adherence and migration (20, 21). Along with these experimental observations, a soluble D1 domain can be detected in urine, likely originating from the vascular compartment (22, 23). However, soluble forms of full-length and truncated (D2D3) CD87 are also found in both blood and urine and are markedly increased during infections, inflammation, and...
cancer (23–28). Mechanistic explanations for the release of such species remain scarce, except for the hydrolysis of the GPI moiety by phosphatidylinositol-specific phospholipases C or D (PI-PLC or PI-PLD) (29–31). Although a juxtacrine proteolytic cleavage of CD87 has also been commonly suggested to occur, the proteinase(s) involved in this process are still unidentified.

PMNs are major effector cells during innate host defense, and their primary recruitment and accumulation into injured tissues appears to determine the subsequent mobilization of other blood leukocytes (32, 33). Activated PMNs can locally release the content of their numerous granules, including the primary/azurophilic granules containing Ser-proteinases such as elastase (EL) and cathepsin G (CG), which have been shown to accumulate at inflammatory foci (33–35). These Ser-proteinases play a crucial role in extracellular proteolytic processes affecting not only matrix components, but also inflammatory effectors such as cytokines or cell membrane receptors, and thus may have immunomodulatory functions in many (patho)physiological situations (33–36). In this context, one striking feature is that they can participate to the chemotaxis of PMNs and mononuclear cells both in vitro and in vivo (32, 35).

The present study provides new insights into the proteolytic regulation of leukocyte CD87 by demonstrating that the two major PMN Ser-proteinases EL and CG cooperate to generate membrane and/or soluble fragments of potential biological importance and to reduce the capacity of inflammatory cells to initiate a uPA-dependent pericellular proteolytic cascade.

Materials and Methods

Reagents

Recombinant human CD87 corresponding to the sequence Leu1–Arg230, lacking the GPI anchor and fused to a C-terminal polyhistidine tag (rhCD87[His]6), was from R&D Systems (Minneapolis, MN). Human leukocyte EL and CG were purified as previously described (37) from human bone marrow PMNs. FITC-conjugated, high-m.w. human uPA was from American Diagnostics (Greenwich, CT). Trpsylin-free bovine pancreatic α-chymotrypsin (αCT; 57 U/mg) and PI-PLC from Bacillus cereus (5100 U/mg) were from Sigma-Aldrich (St. Louis, MO). EL- and CG-free human leukocyte proteinase 3 (PR3), purified from purified human sputum, was obtained from Elastin Products (Owensville, MO). Recombinant eglin C, a potentially serpin enzyme inhibitor for leukocyte Ser-proteinases (38), was provided by Dr. H. P. Schnebli (Novartis, Basel, Switzerland). Human α1-antichymotrypsin (α1ACT), a serum-type plasma inhibitor specific for CG (34, 35), was from Calbiochem (Merck Eurolab, Darmstadt, Germany), whereas N-methoxysuccinyl-Ala-Ala-Pro-Val chloromethylketone (APAV-CMK), a synthetic inhibitor specific for EL (39), was from Sigma-Aldrich. Two synthetic peptides mapping the human CD87 sequences Ser24–Cys59 within the D1-D2 linker domain and Asn72–Gly80 at the C terminus of D3, respectively (1, 30), were prepared with >95% purity by Eurogentech (Seraing, Belgium); the 15-mer Ser-Gly-Arg-Ala-Val-Thr-Thr-Ser-Arg-Ser-real-Arg-Leu-Glu-Cys (SGRAVTYSRSLRC, hereafter designated as the D1-D2 peptide) and the 12-mer Asn-His-Pro-Asp-Leu-Asp-Val-Gln-Tyr-Arg-Ser-Gly (NHPDLDVQYRSG, hereafter designated as the D3 peptide).

Abs used in this study were as follows: mouse mAbs 3931 and 3932, both IgG1 previously referred to as IIIF10 and IID7 (40) and recognizing the amino acid sequences 52–60 of the D1 domain and 125–132 of the D2 domain of CD87, respectively, and mouse mAb 3936, an IgG2a originally described as clone F13/B10 against the Mo3 antigen (29, 41), reacting with an undetermined epitope in human CD87, all from American Diagnostics; nonspecific mAb of the IgG1 and IgG2a subclasses (Ancell, Bayport, MN); His-probe (H-15), a rabbit polyclonal Ab against the His6 peptide tag (Santa Cruz Biotechnology, Santa Cruz, CA); the FITC-conjugated F(ab′)2 goat Ab against mouse IgG (DAKO, Glostrup, Denmark); and HRP-conjugated Abs against mouse or rabbit IgG (ImmunoPure; Pierce, Rockford, IL).

Cell culture reagents were from Life Technologies (Paisley, U.K.), except for heat-inactivated FCS from HyClone Laboratories (Logan, UT), whereas chemicals for cell solubilization, SDS-PAGE, and protein transfer were from Sigma-Aldrich or Bio-Rad (Hercules, CA).

Cells

U937 cell culture, differentiation, and membrane biotinylation. The U937 human promonocytic cell line (42) (CRL-1593.2, American Type Culture Collection, Manassas, VA) was grown as recommended by the supplier, in the presence of 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone (Life Technologies). Before all experiments reported hereafter, U937 cells at 0.5 × 106 cells/ml were cultured for 48–72 h in the presence of 15 nM PMA (Sigma-Aldrich) to differentiate cells into monocytes/macrophages (3, 43), with >70% of cells becoming adherent to plastic and showing approximately 5-, 10-, and 90-fold increases in the expression of the membrane Ags CD14, CD11b/CD18, and CD87, respectively, as judged by FACS analysis (data not shown). Adherent U937 cells were detached by incubation (15 min, 25°C) with HBSS containing 1 mM EDTA, followed by neutralization with 1 mM of both CaCl2 and MgCl2, and two washings at 25°C in HBSS with centrifugations at 530 × g for 15 min. When needed, biotinylation of cell surface molecules was performed essentially as previously reported (44), using sulfo-succinimidyl-6-(biodiamino)hexanenio (EZ-Link Sulfo-NHS-LC-biotin; Pierce) at 1 mg/ml in suspensions of 2.5 × 106 cells/ml in PBS (pH 8.0) containing 1 mM of both CaCl2 and MgCl2, and incubation for 30 min at 25°C. Cells were then diluted with PBS containing 50 mM glucose to quench unreacted biotin and were washed twice in HBSS as above. Either biotinylated or nonbiotinylated cells were finally resuspended in HBSS at 1–2.5 × 106 cells/ml.

Isolation of human blood monocytes. Monocytes were isolated from peripheral blood obtained from human volunteers with their informed consent (Etablissement Français du Sang), as previously described (45). Isolated cells (>95% PMNs) were resuspended at 5 × 106 cells/ml in HBSS supplemented with 1.3 mM CaCl2 and 1 mM MgCl2. Degranulation was obtained by exposing cells to 5 μg/ml cytochalasin B (Sigma-Aldrich) for 5 min under mild stirring, then to 0.5 μM IMLP (Sigma-Aldrich) for another 5 min (47), and then cell suspensions were rapidly cooled to 4°C. Proteinase-enriched, PMN-conditioned media (PMN-CMs) were obtained by pelleting cells at 1700 × g for 10 min at 4°C and then clearing the supernatants from cell debris by centrifugation at 18,000 × g for 15 min at 4°C. PMN-CMs were stored at −80°C. For preparation of control media, cytochalasin B was omitted during incubation (47). Enzymatic activities of EL and CG were measured in PMN-derived media through hydrolysis of specific synthetic substrates as previously described (45), using serial dilutions of each purified enzyme to derive calibration curves.

Exposure of recombinant CD87 or CD87-expressing cells to proteases

Exposure to purified proteins. rhCD87[His]6, adjusted to 1 μg/ml (20 nM) in 50 mM Tris, 100 mM NaCl (pH 7.4) either was mixed with varying concentrations of purified EL or CG or was left untreated as control. After incubation for 5–30 min at 37°C, enzymatic activities were neutralized by addition of 50 μg/ml (6 μM) efgin C, and samples were immediately solubilized for 5 min at 100°C in the presence of 2% (w/v) SDS and 5 mM N-ethylmaleimide (ESDS/NEM).

Suspensions of biotinylated or nonbiotinylated U937 cells or human blood monocytes in HBSS received 1 mM of both CaCl2 and MgCl2, and then varying concentrations of purified leukocyte proteases were added and incubation continued at 37°C for 5–30 min, before enzymes were blocked with 6 μM efgin C. In some experiments, additional suspensions were incubated with 0.25–5 U/ml PI-PLC for 30 min under similar conditions. A fraction of each suspension was kept at 4°C for FACS analysis. Cells in the remaining suspensions were pelleted by centrifugation at 530 × g for 15 min at 4°C and then were solubilized (see Immunoblot analysis of recombinant or cellular CD87). Cell supernatants were collected and further centrifuged at 18,000 × g for 30 min at 4°C to eliminate cell debris before being solubilized with SDS/NEM.

Exposure to activated PMN-conditioned medium. rhCD87[His]6 was adjusted at 1 μg/ml in PMN-derived media prepared as described above, whereas U937 cells or human blood monocytes were resuspended at 1 × 106 cells/ml in these media after the last washing centrifugation. In both cases, incubations immediately started for 5–30 min at 37°C and then enzymatic activities were blocked by addition of 6 μM efgin C. An additional cell suspension made in HBSS containing Ca2+ and Mg2+ was incubated under similar conditions with 0.5 U/ml PI-PLC. Samples containing rhCD87[His]6 were incubated at 37°C for 30 min in the presence of 2 μg/ml anti-human CD87 mAb 3936 and biotinylated with 10 μg/ml streptavidin–HRP (Pierce).

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and both cells and supernatants were solubilized as described above, whereas a fraction of each cell suspension was processed for FACS analysis. In some experiments, PMA-CMs received proteinase inhibitors, i.e., 6 μM eglin C, 1 μM α,ACT, or 25 μM AAPV-CMK, 5 min before starting the incubation with rhCD87[His]₆, or cells.

**Immunoblot analysis of recombinant or cellular CD87**

**Cell solubilization and isolation of biotinylated proteins.** After a final centrifugation, cells were resuspended at 2 × 10⁶ cells/ml and solubilized at 4°C for 30 min in a radioimmunoprecipitation assay medium (pH 7.5) containing 1 mM PMFSF, 10 μM aprotinin, 50 mM benzamidine, 100 μM leupeptin, 1.5 μM soybean trypsin inhibitor, and 5 mM NEM. Immunoprecipitations were performed by an in 13,800 × g centrifugation at 4°C for 30 min, and soluble proteins were stored at −80°C. Protein concentrations were measured using the BCA Protein Assay ( Pierce). For SDS-PAGE and immunoblot analysis, proteins were further solubilized with SDS/NEM.

Biotinylated membrane proteins were separated from nonbiotinylated proteins by incubating 50–100 μg of solubilized total proteins with 40 μl of a half-diluted slurry of NeutraAvidin-agarose beads (Pierce) for 1 h at 4°C in a final volume adjusted to 250 μl. After four washings in 10 mM Tris, 3 mM EDTA, 150 mM NaCl, 1% (v/v) Triton X-100 (pH 6.8), biotinylated proteins were eluted by solubilization in 0.1 ml of the same medium without Triton X-100 and containing SDS/NEM and heating for 5 min at 100°C.

**SDS-PAGE and immunoblot analysis.** rhCD87[His]₆ (15 ng) or cell proteins (10 μg) were separated by SDS-PAGE as previously described (36), with calibration using the Kaleidoscope Prestained Standards (Bio-Rad; Mlow range, 7,600–216,000) for Mlow determination. When required, disulfide bonds were reduced before electrophoresis by adding 5% (v/v) 2-ME and heating for 5 min at 100°C. After SDS-PAGE, proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA). Membranes were first incubated for 1 h in TBST (pH 7.6) containing 0.5% (w/v) dried skimmed milk, and then were incubated for 1 h with the primary Ab in the same medium, washed in TBST, and finally incubated for 1 h with the HRP-coupled secondary Ab. After washings, HRP activity was detected by incubation at 4°C for 1 h in 0.1% (v/v) 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich), and hydrogen peroxide was added as a control

**FACS analysis**

After exposure to enzymes, U937 cells or human blood monocytes were washed in HBSS-BSA, and both cells and supernatants were solubilized as described above, whereas a fraction of each cell suspension was processed for FACS analysis. In some experiments, PMN-CMs received proteinase inhibitors, i.e., 6 μM eglin C, 1 μM α,ACT, or 25 μM AAPV-CMK, 5 min before starting the incubation with rhCD87[His]₆, or cells.

**Matrix-assisted laser desorption ionization (MALDI)-time of flight mass spectrometry (MS) analysis**

Lyophilized samples were diluted in 10 μl of 0.1% (v/v) trifluoroacetic acid and desalted using μ-C18 ZipTips (Millipore) according to the procedure recommended by the manufacturer. Peptides were eluted directly on the MALDI sample plate with 1 μl of a 6-fold dilution of α-cyano-4-hydroxy-cinnamic acid in acetoni-trile/water/trifluoroacetic acid 70/30/ 0.1% (v/v) (all chemicals from Sigma-Aldrich). After air drying of the samples, MALDI-MS measurements were taken on a Voyager-DE STR spectrometer (Applied Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm). Mass spectra were acquired in the reflector and delayed extraction modes using an acceleration voltage of 20 kV, a grid voltage of 76%, and a delay extraction of 150 ns. Each mass spectrum was an average of 250–500 laser shots. Masses were measured using close external calibration with a mixture of four peptides (des-Arg1-bradykinin, monoisotopic mass [M+H]⁺: 904.4681; angiotensin 1, [M+H]⁺: 1296.6853; neurotensin, [M+H]⁺: 1472.9175; adrenocorticotropic hormone fragment 18–39, [M+H⁺]: 2465.1989). Identification of the truncated peptides was conducted with GPMW software, version 6.0 (Lighthouse Data, Odense, Denmark), in regard to the D1-D2 and D3 sequences. Search was performed using monoisotopic masses with a mass tolerance of 0.1%.

**Expression of data**

Results are expressed as mean ± SEM for the indicated number of experiments or measures performed independently.

**Results**

Recombinant human CD87 is cleaved within the D1-D2 linker sequence by purified EL or CG and at its C terminus by CG

The proteolytic activity of EL and CG on the urokinase receptor was first evaluated on the polyhistidine-tagged, recombinant human CD87. After exposure to enzymes, quantitated immunoblotting analysis indicated that rhCD87[His]₆ was similarly altered by either one of the proteinases at low concentrations, i.e., 50–100 nM (Fig. 1, A and B). While the amount of full-length, three-domain rhCD87[His]₆ with M₉, 54,200 ± 3,400 (n = 8) decreased, two molecular species were produced, one with M₉, 38,100 ± 2,000 only recognized by the anti-D2 mAb 3932 and the shortest for the D2D3 and D1 domains, respectively (40, 49). Release of D1 from rhCD87[His]₆ was observed whether intramolecular disulfide bonds were reduced or intact, indicating that cleavage occurred within the D1-D2 linker sequence. EL at concentrations higher than 0.25 μM induced a further degradation of D2D3 and even more efficiently of D1, whereas a further proteolysis of only D2D3 was observed with CG, with D1 accumulating up to 1 μM of enzyme (Fig. 1, A and B). None of the Abs could detect any D1- or D2D3-derived subfragments, indicating a rapid and extensive degradation of these domains in the presence of high enzyme rhCD87[His]₆ species could be observed in the initial untreated material (Fig. 1, A and C, lane 1), likely resulting from coexpression with the full-length protein in the transfected cells, as observed in many cell types expressing CD87 (15, 22, 50, 51) (see next section).

In addition, we examined whether EL or CG was active on the C-terminal region of CD87. For this, proteinase-treated rhCD87[His]₆ was assayed by immunoblotting for the presence of the C-terminal tag epitope. As shown in Fig. 1C, intact nontreated rhCD87[His]₆ (lane 1) produced signals of similar intensities whether reacted with the anti-D2 mAb (upper panel) or the anti-[His]₆ Ab (lower panel). Exposure to 0.5 μM EL (lane 3) resulted,
as expected, in a total conversion of the full-length molecule into D2D3, which still showed an equal reactivity with the anti-D2 and anti-[His]₆ probes. The same concentration of CG (0.5 μg/ml) was exposed at 37°C to 0.05, 0.1, 0.25, 0.5, or 1 μM CG for 30 min or EL for 15 min (lanes 2–6 and 7–11, respectively) or was left untreated for 30 min (lane 1). Proteins (15 ng) were then separated by SDS-PAGE on 10% acrylamide gels under reducing conditions and finally were immunoblotted using the anti-D2 mAb 3932 at 0.05 μg/ml (upper panel) or the anti-D1 mAb 3931 at 1 μg/ml (lower panel). Luminescent signals associated with each molecular species were recorded with a charge-coupled device camera for quantification. Shown are portions of the resulting digitized images corresponding to the location of the relevant Ags. A. Quantitative evaluation of signals associated with each molecular species resulting from the exposure of rhCD87[His]₆ to increasing concentrations of EL (upper graph) or CG (lower graph), expressed as the percentage of the maximal signal obtained for one species within a given experiment. Each data point is the mean ± SEM of three independent experiments. C, rhCD87[His]₆ was exposed to 0.5 μM CG or EL (lanes 2 and 3) or was left untreated (lane 1) for 30 min, except for EL (15 min), and then proteins were separated by SDS-PAGE on 10% acrylamide gels under reducing conditions. Immunoblotting used either the anti-D2 mAb (upper panel) or the anti-[His]₆ His-probe Ab to label the C-terminal polyhistidine tag (lower panel) at 0.05 and 0.1 μg/ml, respectively. Portions of the films corresponding to the location of the relevant Ags, representative of three different analyses, are depicted.

EL cleaves membrane CD87 on monocytic cells preferentially within the D1-D2 linker sequence, whereas CG preferentially cleaves a juxtamembrane sequence

**FACS analysis.** The differentiated U937 cell line was used as a model of CD87-expressing monocyte/macrophage-type cells (3, 43, 52). The proteolytic activity of purified EL and CG on membrane CD87 was first explored by FACS analysis, using the already mentioned anti-D1 mAb 3931 and anti-D2 mAb 3932 as well as mAb 3936, which recognizes a still unknown conformational epitope (41, 48). Exposure of cells to EL for 15 min (Fig. 2, upper left panel) resulted in a progressive decrease in binding of the anti-D1 mAb from 0.1 to 0.5 μM of enzyme, with an ~75% maximal reduction. By contrast, binding of the anti-D2 mAb and of mAb 3936 did not markedly decrease up to 1 μM of enzyme. Time course analysis indicated that binding of 3931 was already reduced by ~65% after 5 min of exposure to 0.2 μM EL, whereas binding of 3936 remained unchanged over a 30-min period (data not shown). Exposure of cells to CG for 30 min had clearly different consequences because binding of all three mAbs was progressively decreased from 0.25 to 1 μM of enzyme, with an ~80% maximal reduction (Fig. 2, upper right panel). Time course analysis with 0.5 μM CG showed that a 30-min exposure to the enzyme was required for maximal effects (data not shown). Exposure of U937 cells to either enzyme did not increase the number of propidium iodide-positive dead cells. The modifications in CD87 expression resulting from cell exposure to the leukocyte Ser-proteinases were compared with those produced by bacterial PI-PLC, which hydrolyze the GPI structure between the inositolphosphate ring and the diacylglycerol moiety (53). Experiments not illustrated indicated that exposure of cells to 0.5 U/ml PI-PLC for 15 min resulted in maximal effects on GPI-anchored membrane proteins such as CD14 and CD87. Under these conditions, PI-PLC maximally reduced CD87-associated epitope display by 44 ± 5%, 47 ± 2%, and 40 ± 10% (n = 3) when using mAb 3931, 3932, or 3936, respectively, in agreement with previous observations (29, 30).

The evaluation of a leukocyte proteinase-dependent regulation of CD87, as observed on the differentiated U937 cell line, was extended to primary mononuclear phagocytes. For this, we used preparations of PBMCs highly enriched in monocytes, which express CD87 (54). As judged by FACS analysis using the anti-D1 and anti-D2 mAbs (Fig. 2, lower panels), exposure of blood monocytes to EL up to 1 μM for 15 min resulted in an ~50% reduction in display of the D1 domain, whereas display of the D2 domain was unaffected. Conversely, exposure to CG in the same range of concentrations for 30 min led to a reduction in display of both the D1 and D2 domains, to ~50% of that observed on control untreated cells. Thus, the susceptibility of membrane CD87 expressed on human blood monocytes to specific modifications by EL or CG appears to be similar to that described using differentiated U937 cells.

**Immunoblotting analysis.** Although CD87 is easily detected in whole cell extracts by immunoblotting using domain-specific mAbs (17, 40), accurate evaluation of its proteolytic modifications.
on plasma membranes was hampered by the presence of an intracellular pool (44, 55) inaccessible to proteinases. This problem was solved by cell surface biotinylation, which allows for specific analyzing of membrane proteins. As illustrated in Fig. 3A, when biotinylated membrane proteins obtained from control, proteinase-ununtreated U937 cells are analyzed by immunoblot using the anti-D2 mAb 3932 (lane 1), membrane CD87 appears as a major upper species with $M_r$ 65,100 ± 1,400 ($n = 7$), which is also reactive with the anti-D1 mAb 3931 (data not shown) and corresponds to the membrane full-length (mD1D2D3) CD87, whereas a minor lower species with $M_r$ 49,700 ± 2,000 reactive only with the anti-D2 mAb represents a truncated, two-domain (mD2D3) form, as previously observed (15, 22). It must be noted that a high level of D2 mAb represents a truncated, two-domain (mD2D3) form, as lower amounts compared with EL-derived samples.

Immunoblotting analysis of membrane CD87 from biotinylated U937 cells exposed to EL or CG completed the data obtained in FACs analysis. Thus, EL induced, up to 0.5 μM, a proteolytic transition from the mD1D2D3 to the truncated mD2D3 species (Fig. 3A, lanes 2 and 3), which accumulated at the cell surface and remained barely affected by the enzyme up to 3 μM (data not shown). By contrast, exposure of cells to CG from 0.25 to 1 μM (Fig. 3A, lanes 4-6) resulted in the disappearance of both the full-length and truncated CD87 species. No fragments with $M_r$ lower than that of mD2D3 could be detected, and similar results were obtained when considering proteins with intact disulfide bonds (data not shown). In parallel, analysis of the cell supernatants indicated that although exposure to EL resulted in the release of only trace amounts of soluble D2D3 (sD2D3), exposure to CG induced the accumulation of both sD1D2D3 and sD2D3, with $M_r$ values of 63,300 ± 1,600 and 50,700 ± 2,000 ($n = 6$), respectively (Fig. 3B, upper panel). Similar features were observed when supernatants were analyzed under nonreducing conditions (data not shown). Immunoblotting with the anti-D1 mAb revealed a rather heterogeneous material with a mean $M_r$ of ~17,500, corresponding to soluble D1 (sD1) in the supernatants from EL-treated cells (Fig. 3B, lower panel), which disappeared with higher enzyme concentrations (data not shown). Supernatants derived from CG-treated cells also showed the presence of sD1 species with similar $M_r$, but in lower amounts compared with EL-derived samples.

The two enzymes thus exert contrasted effects on membrane CD87, with EL proteolytically active mostly on the D1-D2 linker sequence and within D1, and to a much lesser degree on the C-terminal part of the molecule, whereas CG is essentially active within this latter juxtapamembrane domain of the receptor, with a weaker activity on the D1-D2 region.

**EL and CG secreted from PMNs cooperate for the generation of a major soluble D2D3 species**

Evaluation of combined effects of EL and CG on CD87 structure was performed by preparing conditioned milieus from degranulated human blood PMNs, which comprise essentially EL and CG as proteinases (34). In agreement with previous data (36, 46), such PMN-CMs contained concentrations of active EL approximately twice those of CG, respectively 0.39 ± 0.07 μM and 0.20 ± 0.05 μM ($n = 6$). Activation of PMNs with IMLP in the absence of cytochalasin B provided control milieus with no detectable EL or CG activities. As illustrated in the upper and middle panels of Fig. 4, complete cleavage of rhCD87[His]$_6$ occurred within 5 min of incubation in the proteinase-rich milieu (lane 2), generating a major D2D3 form with $M_r$ 38,700 ± 1,000 ($n = 2$), which remained...
stable up to 30 min of incubation, and a free D1, which was further proteolyzed and became undetectable by 30 min. Blocking of leukocyte Ser-proteinase activities with eglin C totally abolished the conversion of rhCD87[His]6 into D2D3 (lane 1). The EL-specific chloromethylketone inhibitor AAPV-CMK, which completely inhibited EL activity in PMN-CMs, drastically reduced formation of D2D3 and degradation of free D1 (lane 3). By contrast, specific inhibition of CG up to 85% with α1-ACT had limited effect on the conversion of the full-length CD87 into D2D3 and on degradation of D1 (lane 4). Immunoblotting with the anti-[His]6 Ab (Fig. 4, lower panel) showed a loss of the C-terminal histidine tag upon incubation with PMN-CMs (lane 2, 30-min incubation). This proteolytic event was impaired by the presence of eglin C (lane 1) and was still observed when EL was specifically blocked (lane 3), but was considerably reduced when CG was inhibited (lane 4). In all cases, incubation in the control PMN-derived milieu had no effect on the structure of CD87 (data not shown).

We then tested the activity of PMN-CMs on cell membrane CD87 (mCD87). Exposure of U937 cells to this milieu for 30 min at 37°C followed by FACS analysis indicated that binding of mAbs 3931, 3932, and 3936 was reduced to 13 ± 6%, 46 ± 5%, and 63 ± 6% (n = 4), respectively, whereas exposure to the control milieu had no effect on epitope display. The decrease in mAb binding could be detected as early as 5 min after cell exposure to PMN-CMs (data not shown). Immunoblotting analysis of proteins in the cell fraction confirmed that, by 30 min of incubation in PMN-CMs, mCD87 had been extensively proteolyzed (data not shown). More interestingly, analysis of cell supernatants (Fig. 5A) showed that a major species with Mr 52,000 ± 2,100 (n = 3) had been released, which was reactive with the anti-D2 mAb (upper left panel, lane 2), but not with the anti-D1 mAb (data not shown), thus corresponding to sD2D3. Immunoblotting with the anti-D1 mAb showed no detection of free D1 (Fig. 5A, lower left panel). Incubation in control PMN-derived milieu resulted in barely detectable material, except for traces of an sD1 species (lane 1). The release of sD2D3 was inhibited when eglin C was present during the incubation with PMN-CMs with, again, only small amounts of sD1 being detected (lane 3). When EL was inhibited by AAPV-CMK (lane 4), cell supernatants displayed both sD1D2D3, as confirmed by its reactivity with the anti-D1 (data not shown), as well as the anti-D2 mAb and sD2D3, with higher amounts of sD1 being clearly detectable with Mr 14,900 ± 1,500 (n = 3). By contrast, when CG was inhibited with α1-ACT (lane 5), only minute amounts of sD2D3 could be detected, and no sD1. Importantly, similar results were obtained when cell supernatants were analyzed under nonreducing conditions (Fig. 5A, upper right panel). To further assess the specific nature of the CD87 fragment released upon exposure of cells to PMN-derived proteinases, U937 cells were exposed in parallel to 0.5 U/ml PI-PLC for 30 min, which resulted in the release of ~50% of the membrane-associated CD87 (see previous section). Immunoblot analysis of cell supernatants, whether reduced or nonreduced, using the anti-D2 mAb indicated that PI-PLC releases species reflecting the initial membrane-associated CD87, i.e., mostly sD1D2D3 and only trace amounts of sD2D3 (Fig. 5B, lanes 2), as opposed to the marked and only sD2D3 observed in supernatants of PMN-CM-treated cells (lanes 3).

Total inhibition of the proteolytic processing of CD87 by an inhibitor specific for EL and CG, i.e., eglin C (38), suggested that the proteolytic activity directed at CD87 in PMN-CMs was restricted to these two proteinases. However, PR3 is yet another Ser-proteinase stored in and secretable by PMNs upon degranulation, together with EL and CG (34, 57). Thus, we evaluated the potential activity of PR3 on CD87 by exposing U937 cells to purified human PR3 at concentrations up to 1 μM for up to 45 min at 37°C. Subsequent FACS analysis of PR3-treated cells showed no decreased display of CD87-associated epitopes recognized by mAb 3931, 3932, or 3936 (data not shown), thus excluding that PR3 participates in the proteolytic processing of CD87 exposed to PMN-CM.

Thus, the natural combination of EL and CG results in a cleavage of rhCD87[His]6 as well as of mCD87 corresponding to the additive effects of each purified enzyme used separately at a concentration similar to that measured in PMN-CMs (Figs. 1 and 3), i.e., a rapid release and further proteolysis of D1 essentially due to EL and an equally rapid cleavage at the C terminus of D3 mostly due to CG. Cooperation of the two proteinases acting on monocytic cells ultimately results in the release of a major, quite stable D2D3 species.

**Determination of cleavage sites for EL and CG in CD87-related peptides**

Cleavage sites for EL and/or CG within identified proteinase-sensitive domains in CD87 were approached through MALDI-MS analysis of the proteolytic fragmentation of two synthetic peptides mimicking the D1-D2 linker sequence, as well as the D3 C-terminal domain ahead of the last cysteine residue engaged in disulfide bonding (1, 2), respectively. For each MALDI-MS analysis of peptides submitted or not to the action of the two selected proteinases, peptide identifications were achieved through the use of GPMAW software. Results are summarized in Table I and are as follows for the D1-D2 peptide: 1) exposure to aCG, taken as a reference Ser-proteinase active on CD87, resulted, as expected from previous data (2, 18), in a single cleavage after a Tyr residue corresponding to Tyr87 in human CD87; 2) exposure to EL resulted in a rapid...

**FIGURE 5.** Cooperative cleavage of membrane CD87 on U937 cells and release of truncated D2D3 species by EL and CG secreted from PMNs. A, Nonbiotinylated U937 cells were exposed for 30 min at 37°C to the control (lane 1) or proteinase-enriched (lanes 2–5) PMN-CMs used in Fig. 4. Incubations in fractions of the proteinase-enriched PMN-CMs were also performed in the presence of Ser-proteinase inhibitors: 6 μM eglin C (lane 3), 25 μM AAPV-CMK (lane 4), or 1 μM α1-ACT (lane 5). Cell supernatants were then isolated from cells and their proteins were separated by SDS-PAGE as described in the legend to Fig. 3B, under reducing (left panels) or nonreducing (right panels) conditions. Immunoblotting was performed using the anti-D2 (upper panels) or anti-D1 mAb (lower panel) as described in the legend to Fig. 1. B, Nonbiotinylated U937 cells were exposed for 30 min at 37°C to the control (lane 1) or proteinase-enriched PMN-CMs (lane 3) or to 0.5 U/ml PI-PLC (lane 2), and cell supernatants were immunoblotted with the anti-D2 mAb as described in the legend. Portions of films corresponding to the location of the relevant Ags, representative of three independent experiments, are shown.
cleavage (within 1 min) after a Val residue corresponding to Val85, thus confirming a previous observation (2); 3) exposure to CG resulted, within 15 min, in cleavages after Tyr residues corresponding to Tyr87, as for αCT, and to Tyr92; and 4) exposure to a combination of EL and CG rapidly produced the EL-derived fragment corresponding to cleavage after Val85, with an \([M+H]^+\) value of 1276.6448, which was then converted, through cleavage by CG after Tyr92, into the heptapeptide TYSRSRY, which remained detectable up to 30 min of proteolysis.

Exposure of the D3 peptide to CG resulted in its complete cleavage, within a few minutes, after residues corresponding to Arg83 and to Tyr92 (or less likely Asn272) in human CD87 (1, 2). By contrast, only longer exposure to EL (30 min) resulted in some cleavage after the Val residue corresponding to Val85. Combination of the two proteinases for 30 min resulted in a mixture of the three cleavages.

**Binding of uPA to monocytic cells is drastically reduced by EL and CG**

Membrane CD87 is a major binding site for uPA, which the N-terminal domain can interact with several discrete sequences located within all three domains of CD87 (2, 58). Because EL and CG have the capacity to proteolytically affect some of these domains, we next investigated how these proteinases could affect the interactions of uPA with its receptor, using a FITC-coupled uPA binding assay (48). When U937 cells were exposed at 4°C to increasing concentrations of FITC-uPA, FACS analysis indicated that a near saturating binding was observed for a ligand concentration of 50 nM. Specificity of the interaction was further established through the observations that fluorescence associated with undifferentiated, i.e., CD87-negative, cells was reduced to that of the nonspecific binding seen with an irrelevant FITC-conjugated protein, whereas binding performed on differentiated cells in the presence of a 20-fold molar excess of unlabeled uPA resulted in similar nonspecific fluorescence.

Exposure of cells to EL for 15 min or to CG for 30 min resulted in an enzyme concentration-dependent reduction of their capacity to bind FITC-uPA, with EL appearing slightly more efficient than CG, but in both cases maximal inhibition reached >95% (Fig. 6). Similarly, cells incubated for 30 min in the proteinase-enriched PMN-CMs exhibited a similar reduction of binding of FITC-uPA (Fig. 6), whereas a control PMN-derived milieu had no effect (data not shown). For comparison, incubation of cells with 0.5 U/ml PI-PLC for 30 min reduced the binding capacity to 26 ± 2% of the control value (n = 2). In the case of EL and PMN-CM, reduction in the binding of FITC-uPA appeared to be fairly well related to that of the anti-D1 mAb 3931 (Fig. 6). It must be noted that the conformational, yet unknown epitope recognized by mAb 3936, which interferes with uPA binding and has been extensively used in immunohistochemistry studies (41, 48), appears to be unrelated to expression of domain D1.

**Discussion**

Data reported here establish that the PMN-derived Ser-proteinases EL and CG cooperate for the proteolytic regulation of CD87 on monocyteic cells. On one hand, EL is highly efficient at removing domain D1 while leaving most of the D2D3 truncated receptor associated with the membrane. In contrast, CG also has the capacity to release D1, but actually appears more efficient in the proteolysis of the CD87 C terminus, resulting in the release of both full-length and truncated CD87 species. As depicted in Fig. 7, combination of the two Ser-proteinases resulting from degranulation of PMNs ultimately leads to the accumulation of soluble D2D3, whereas D1 is not detected due to further proteolysis by EL. The D1-D2 linker sequence covers residues Cys76-Cys89 in human CD87 (1, 2, 18), and cleavage sites for various proteinases have been shown to include the peptide bonds after Arg83 and Arg89 for uPA and potentially for plasmin, Val85 for EL, Thr86 for various MMPs, and Tyr87 for αCT (2, 15, 16, 18). CG releases a free D1 domain with \(M_r \sim 16,000\), indistinguishable from that generated by other proteinases (Ref. 59 and data not shown) and, in agreement with its chymotrypsin-like Ser-proteinase activity (34, 35), we have identified likely cleavage sites for CG within the D1-D2 linker sequence as peptide bonds after Tyr87 and/or Tyr92, while confirming that EL can efficiently cleave after Val85. Regarding the juxtamembrane cleavage site(s), the release by CG of full-length and truncated CD87 species from the cell surface suggests that cleavage of D3 must occur below the last cysteine residue (Cys271) engaged in disulfide bonding (2), and indeed we determined potential cleavage sites as the peptide bonds after Val85 and Arg83, corresponding to cleavage after Tyr87 and/or Tyr92, respectively.

**Table 1. MALDI-MS analysis of proteinase-induced fragmentation of CD87-related peptides D1–D2 and D3**

<table>
<thead>
<tr>
<th>Enzymatic Treatment</th>
<th>([M+H]^+)</th>
<th>Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1–D2 peptide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1788.8780</td>
<td>SGRAVTYSSRSLRUC</td>
</tr>
<tr>
<td>αCT</td>
<td>1012.4828</td>
<td>SRSRYLECH</td>
</tr>
<tr>
<td>EL</td>
<td>1276.6240</td>
<td>TYSRSRYLCEC</td>
</tr>
<tr>
<td>CG</td>
<td>1444.7610</td>
<td>SGRAVTYSRSRRY</td>
</tr>
<tr>
<td>EL + CG</td>
<td>1276.6002</td>
<td>TYSRSRYLCEC</td>
</tr>
<tr>
<td></td>
<td>932.4584</td>
<td>TYSRSRY</td>
</tr>
<tr>
<td>D3 peptide</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>1441.6937</td>
<td>NHPPDLVQYRS</td>
</tr>
<tr>
<td>EL</td>
<td>851.4078</td>
<td>NHPPDLV</td>
</tr>
<tr>
<td>CG</td>
<td>1298.6305</td>
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</tr>
<tr>
<td>EL + CG</td>
<td>1142.5208</td>
<td>NHPPDLVQY</td>
</tr>
</tbody>
</table>

\(a\) Peptides (65 μM) were exposed for 1–30 min at 37°C to proteinases in peptide/enzyme molar ratios varying from 10 to 1000 or to a control proteinase-free medium.

\(b\) Values for the \([M+H]^+\) monoisotopic ions observed in an experiment representative of four for the D1–D2 peptide and two for the D3 peptide.

\(c\) Truncated peptides were deduced from the masses fitted to the amino acid composition of the original D1–D2 and D3 peptides using OMPMAW software.

**FIGURE 6.** Reduction of the capacity of U937 cells exposed to purified EL or CG, or to their combination, to bind uPA. U937 cells were exposed to various concentrations of purified EL or CG or to the proteinase-enriched PMN-CMs or were incubated under control conditions as described in the legends to Figs. 2 and 5, respectively. Cells were then incubated for 1 h at 4°C with 50 nM FITC-uPA or with mAb 3931 (anti-D1) or 3936, both at 5 μg IgG/ml, and binding was assessed by FACS analysis as described in Materials and Methods. Specific mean fluorescence intensity was expressed as the percentage of the value obtained for cells incubated in the absence of proteinase or in the control PMN-derived milieu. Results are shown as means ± SEM of three independent experiments.
after Asn 272. This assumption is supported by the fact that we were analyzing CG-treated rhCD87[His]6 by SDS-PAGE using Tris-lysine tricine gels, indicating that the peptide bearing the tag had a molecular mass lower than 1500 Da, the limit of separation of such gels (data not shown). Although it appears to be much less efficient, EL may also cleave the C-terminal domain of CD87 after Val 278, which could explain a very slight release of soluble D2D3 species from cells (Figs. 3B, lanes 2 and 3; and Fig. 5A, lane 5).

The novel molecular mechanism that has been described allowing two leukocyte proteinases to complement each other to reduce cell surface expression of D1 and to generate membrane-bound and soluble D2D3 species sheds some light on previous observations and has potential major (patho)physiological implications. Thus, the presence of soluble forms of CD87, mostly full-length or D2D3 species and more scarcely D1, is a common feature of body fluids such as blood plasma or urine obtained from normal subjects (22, 23), and concentrations of these products are elevated in several pathologies such as solid tumors (5, 24, 26), bone marrow malignancies (23), and more particularly local or systemic infectious and inflammatory disorders (25, 27, 28). Accordingly, cells in culture can release soluble CD87, as well as D1 and D2D3 fragments (22, 60, 61), and shedding of CD87 is increased upon cell activation (19, 28, 61), including exposure of CD87-expressing endothelial cells to isolated leukocytes (60). Although release of free D1 has been ascribed to the presence of active uPA, plasmin, and/or various MMPs in the cell environment (15, 19), release of full-length or truncated CD87, which must implicate juxtamembrane hydrolysis, has received little explanation yet, except for the hydrolysis of the GPI moiety by bacterial PI-PLC or host PI-PLD activities (29–31). Proteolysis of the C-terminal region of CD87 has been suggested to be a potential mechanism (19, 60, 61), although none of the proteinases considered so far has been shown to cleave membrane CD87 outside the D1-D2 linker sequence (16, 17, 19). We provide here the first demonstration of a secretable Ser-proteinase, i.e., CG, that exerts its enzymatic activity essentially toward the CD87 C terminus. Our observation offers an important alternative for the release of CD87, which may prove to be particularly operative in situations of intense PMN recruitment and accumulation at inflammatory foci. Support to this assumption can be found in the observation 1) that the levels of soluble CD87 in inflammatory exudates taken from septic patients appear to correlate with the local accumulation of PMN (25), and 2) that free leukocyte Ser-proteinase activities in the range or even exceeding the concentrations of purified enzymes used in the present study can be measured in biological fluids under certain pathological situations, such as those observed in the airspaces of patients with bronchiectasis or cystic fibrosis (35, 62, 63).

Removal of at least D1 from membrane CD87 expressed on inflammatory cells such as monocytes/macrophages should have major impacts on cell functions. As shown in the present study, this drastically reduces the capacity of cells to bind uPA (Fig. 7), thus confirming that D1 indeed controls the high-affinity conformation of CD87 for this ligand, although the binding site for uPA is composite and implicates sequences in all three domains (2, 58). Decreased uPA binding very likely results in a reduced pericellular generation of plasmin (2, 4) and, consequently, a reduced activation of several MMPs (6). However, removal of D1 should also profoundly perturb cell adherence and migration 1) by impairing the endocytosis of CD87 and its recycling to the leading edge of migrating cells, which depends on the formation of a trimolecular complex among plasminogen activator inhibitor-1, uPA, and CD87 (20, 2) by abrogating the high-affinity binding of CD87 to the extracellular matrix protein vitronectin (7, and 3) by disrupting the physical and functional interactions with integrins, particularly with the leukocyte major integrin αmβ2 (8–10). Regarding this latter point, it has been shown in several models that CD87-deficient mice present a drastically reduced β2 integrin-dependent recruitment of PMNs and monocytes at sites of experimentally induced infection and inflammation (12, 13). Limited proteolytic truncation of CD87 by leukocyte Ser-proteinases could well be a negative feedback mechanism down-regulating αmβ2-mediated cell functions (8, 9). However, an excessive reduction of the capacity of inflammatory cells to migrate on and adhere to fibrin- and vitronectin-rich provisional inflammatory extracellular matrices and to support pericellular proteolysis could be detrimental for the elimination of such matrices and for proper tissue healing (21, 64, 65).

Finally, generation of membrane or soluble D2D3 species could also be of a major importance in changing the cell phenotype. Indeed, CD87 is endowed with a chemotactic and promigratory activity which can be, for a part, ascribed to the proteolytically driven expression of a new N-terminal sequence after cleavage of the D1-D2 linker domain (11). This sequence must contain the active motif Ser88-Arg-Ser-Arg-Tyr92 (SRSRY) and is chemotactic for monocyctic cells in a nanomolar range of concentrations while remaining inactive in the full-length molecule (11, 66). D2D3 species are expressed on the plasma membrane of various cell types, and this expression is increased after endoproteolysis of the full-length CD87 (15, 17, 19, 50), as shown in this study with monocyctic cells exposed to leukocyte Ser-proteinases. Thus, this process could provide cells with a cis- or trans-acting, membrane-bound chemokine-like structure (11, 20, 21). Maybe more importantly, the combined proteolysis of membrane CD87 by EL after Val85 and by CG acting preferentially at the juxtamembrane D3 domain may release soluble D2D3 molecules bearing the SRSRY motif, whereas an even further possibility is that cleavage by CG after Tyr92, secondary to that after Val85 by EL, will release the heptapeptide TYSRSRY (Fig. 7). Such proteolytic processings of CD87 would produce molecules that might behave as true, fully diffusible chemotactic agents, and thus would participate in the
orchestrated recruitment of leukocytes to an inflammatory site (14, 32), a hypothesis currently under investigation.

Acknowledgments

We thank Viviane Balloy for assistance in the preparation of purified EL and CG, Dr. Anne-France Petit-Berton for kindly providing human blood monocyte suspensions, and Drs. Agustín Valenzuela-Fernández and Thierry Planchnaut for help in quantification of immunoblots.

References


