An Interactive Network of Elastase, Secretases, and PAR-2 Protein Regulates CXCR1 Receptor Surface Expression on Neutrophils*

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Background: IL-8 activates neutrophils through CXCR1. Elastase cleaves CXCR1, but the cellular machinery involved remains poorly understood.

Results: Here we show that secretases and PAR-2 regulate elastase-mediated cleavage of CXCR1.

Conclusion: These studies establish a novel network of elastase, secretases, and PAR-2 that regulates CXCR1 on neutrophils.

Significance: Targeting this network may lead to novel therapeutic strategies in neutrophilic diseases, such as cystic fibrosis.

CXCL8 (IL-8) recruits and activates neutrophils through the G protein-coupled chemokine receptor CXCR1. We showed previously that elastase cleaves CXCR1 and thereby impairs antibacterial host defense. However, the molecular intracellular machinery involved in this process remained undefined. Here we demonstrate by using flow cytometry, confocal microscopy, subcellular fractionation, co-immunoprecipitation, and bioluminescence resonance energy transfer that combined α- and γ-secretase activities are functionally involved in elastase-mediated regulation of CXCR1 surface expression on human neutrophils, whereas matrix metalloproteases are dispensable. We further demonstrate that PAR-2 is stored in mobilizable compartments in neutrophils. Bioluminescence resonance energy transfer and co-immunoprecipitation studies showed that secretases, PAR-2, and CXCR1 colocalize and physically interact in a novel protease/secretase-chemokine receptor network. PAR-2 blocking experiments provided evidence that elastase increased intracellular presenilin-1 expression through PAR-2 signaling. When viewed in combination, these studies establish a novel functional network of elastase, secretases, and PAR-2 that regulate CXCR1 expression on neutrophils. Interfering with this network could lead to novel therapeutic approaches in neutrophilic diseases, such as cystic fibrosis or rheumatoid arthritis.

The chemokine CXCL8 (IL-8) attracts and activates neutrophils through the G-protein-coupled receptors CXCR1 (IL-8RA) and CXCR2 (IL-8RB) to combat pathogens and drive inflammation (1–3). Particularly CXCR1 is involved in antibacterial host defense functions of neutrophils, rendering insights into mechanisms regulating CXCR1 expression essential for understanding and therapeutically targeting neutrophilic immune responses. We found previously that the serine protease elastase inactivates CXCR1 functionality by a complex cleavage mechanism involving the release of the C-terminal moiety of the receptor into the extracellular microenvironment (4), a mechanism disabling anti-bacterial host defense in CF3 lung disease (4, 5). This receptor disintegration process can hardly be explained through the action of a single protease (elastase), particularly as CXCR1 represents a seven-transmembrane G-protein-coupled receptor. Based on our current view, post-translational disintegration of CXCR1 involves at least two distinct molecular events: (i) proteolytic cleavage of an extracellular moiety of CXCR1 by elastase activities and (ii) disintegration of the C-terminal CXCR1 receptor part through a so-far unidentified intracellular machinery. Because the C-terminal moiety of CXCR1 is physically not accessible to exogenous proteases, we hypothesized that elastase, besides its direct proteolytic effects, activates an intracellular downstream network of proteases, which collaborate in the disintegration of the C-terminal CXCR1 moiety.

Human neutrophils express a variety of proteases (6) within preformed intracellular granules and at their cell surface (7), mainly serine proteases (elastase, cathepsin G and proteinase 3), matrix metalloproteases (MMPs), and disintegrin and metalloproteases (ADAM), which activate and interact with each other (8, 9). To dissect the elastase-downstream mechanisms involved in CXCR1 processing, we applied a small molecule screen covering different classes of neutrophil proteases including MMP, ADAM/α-secretases, and γ-secretases fol-

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3 The abbreviations used are: CF, cystic fibrosis; co-IP, co-immunoprecipitation; MMP, matrix metalloprotease; ADAM, disintegrin and metalloprotease; BRE, bioluminescence resonance energy transfer; HBSS, Hanks’ balanced salt solution; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; NCSTN, nicastrin; BSG, basigin; TLR, Toll-like receptor.
lowed by targeted biochemical and immunological analyses. These studies identified a novel interactive network of elastase, secretases, and PAR-2, which regulates CXCR1 surface expression on neutrophils. Unexpectedly, α- and γ-secretases, enzymes originally described to cleave and liberate the amyloid precursor protein, played a key role in this network.

**EXPERIMENTAL PROCEDURES**

**Neutrophil Isolation**—Human neutrophils were isolated as described previously by us (10). In brief, peripheral blood was obtained from healthy volunteers in accordance with the institutional review board and approved by the Ethical Committees of the Ludwig Maximilians University Munich and the University of Tübingen. After ammonium chloride erythrocyte lysis, human neutrophils and peripheral blood mononuclear cells were prepared by Ficoll-Hypaque density gradient centrifugation of heparinized blood from healthy volunteers. Cells were cultured in HBSS or RPMI 1640 (Biochrom) supplemented with 10% FCS, 10 mM HEPES (Sigma), 1.5 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from PAA Laboratories) as indicated.

**Inhibitors**—Used inhibitors, concentrations, and commercial sources are shown in Table 1. Optimal inhibitor working concentrations were determined by (i) using concentrations 3-fold above reported IC50 values, (ii) measuring phorbol 12-myristate 13-acetate-induced MMP activity after inhibitor treatment, and by (iii) analyzing neutrophil apoptosis and necrosis after inhibitor treatments (data not shown).

**Subcellular Fractionation of Neutrophils**—Subcellular fractionation of neutrophils was performed by nitrogen cavitation and sedimentation of the postnuclear supernatant on a four-layer Percoll density gradients, as described previously in detail by Clemmensen et al. (11, 12) and our studies (10, 13). In brief, neutrophils isolated from peripheral blood were resuspended in disruption buffer (100 mM KCl, 3 mM NaCl, 1 mM Na2ATP 3.5 mM MgCl2, 10 mM PIPES, pH 7.2) with a protease inhibitor mixture added as described by the manufacturer (11836153001, Roche Applied Science). Neutrophils were disrupted by nitrogen cavitation at 380 p.s.i. for 5 min and collected in 1.5 mM EGTA. Furthermore, the cavitate was centrifuged at 400 × g for 15 min to remove nuclei and unbroken cells, and the supernatant was added to a Percoll solution with a density of 1.11 g/ml at a ratio of 1:1, resulting in a final density of 1.055 g/ml. Next, 9 ml of the Percoll solution with density 1.03 g/ml were underlayered with 9 ml of the supernatant to create a flotation medium for separation of plasma membranes/cytosol and secretory vesicles. Furthermore, this was underlayered with a two-layer Percoll gradient (9 ml with a density of 1.09 g/ml and with 9 ml of a density of 1.12 g/ml) to separate azurophil, specific, and gelatinase granules. pH was adjusted to 7.0 by HCl. The four-layer gradient was centrifuged 20,000 × g for 40 min, resulting in 5 major bands: the α-band enriched in primary/azurophil granules (marker: myeloperoxidase), the β1-band enriched in secondary/specific granules (marker: neutrophil gelatinase-associated lipocalcin), the β2-band enriched in tertiary/gelatinase granules (marker: gelatinase/MMP9), a γ1-band enriched in secretory vesicles (marker: albumin), and the γ2 band-containing plasma membranes (marker: human leukocyte antigen). Samples were subjected to ELISA analysis or to SDS-PAGE and Western blot analysis. Myeloperoxidase, neutrophil gelatinase-associated lipocalcin, MMP-9, HSA, and human leukocyte antigen were quantified in each fraction by ELISA and used as marker proteins for azurophil granule, specific granules, gelatinase granules, secretory vesicles, and plasma membrane, respectively.

**Western Blot**—Neutrophil fractions were separated on NuPAGE 4–12% BOLT Bis-Tris Plus gels (Invitrogen), and immunoblotting was performed by standard procedures using XCell II blotting chambers (Invitrogen). After blocking, a primary antibody against PAR-2 (Abcam, ab128628) was incubated overnight. Blots were processed by using alkaline phosphatase-conjugated secondary antibodies (DAKO) and ECL solution (GE Healthcare). Semiquantitative analysis was performed with the Quantity One software system (Bio-Rad).

**Flow Cytometry**—Flow cytometric data were obtained on a FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences). Staining was performed following standard procedures. The mAbs used were anti-presenilin-1 and anti-PAR-2. Appropriate isotype controls were used for all applied detection antibodies. For intracellular cytokine, detection cells were preincubated with brefeldin A at 10 μg/ml. After surface staining, cells were fixed with 2% paraformaldehyde and subsequently permeabilized with saponin 0.1% and then stained for intracellular proteins.

**Confocal Microscopy**—For visualization of presenilin-1, the samples were incubated with rabbit anti-human presenilin-1 (Abcam, ab15456 monoclonal) or anti-human CXCR1 antibodies (Santa Cruz, polyclonal). The primary antibodies were detected in confocal laser-scanning microscope by means of a secondary anti-rabbit Alexa Fluor 555 antibody (Invitrogen). DNA was stained with DAPI (Sigma), and concanavalin A Alexa 488 conjugate (Invitrogen) was used for detecting glycoconjugates in the cytoplasm. The specimens were analyzed with a confocal laser-scanning microscope (Olympus IX 51).

**Bioluminescence Resonance Energy Transfer (BRET)**—Interactions between CXCR1 and candidate proteins of the α- and γ-secretase or PAR-2 were tested using BRET where Renilla luciferase (Rluc) serves as donor and yellow fluorescent protein (YFP) serves as the acceptor of energy transfer, occurring when proteins physically interact (14). Plasmids were constructed carrying the coding sequence of candidate proteins fused to Rluc or YFP protein, respectively. BRET experiments were performed as described previously (15). For each interaction HEK293 cells were co-transfected with a total of 0.8 μg of DNA at an acceptor-to-donor ratio of 3:1. For BRET titration experiments that where performed to distinguish a true positive interaction and bystander BRET resulting from random collision, HEK293 cells were co-transfected at increasing acceptor-to-donor ratios with a total of 2 μg of DNA. For BRET titration experiment that were performed to distinguish direct and non-direct interactions, cells were co-transfected at increasing DNA amounts at a constant acceptor-to-donor ratio of 1. All experiments where performed in duplicate and at least two independent experiments. After 24 h, coelenterazine (30 μM) was added to the living cells, and light emission was collected in a 96-well microplate luminometer LUMIstar OPTIMA (BMG
Labtech) for 10 s at 475 nm (Rluc signal) and 535 nm (BRET signal). BRET ratio was calculated based on $R = I_d / I_a - cf$, where $R$ is the BRET ratio, $I_d$ is the intensity of light emission at 535 nm, $I_a$ is the intensity of light emission at 475 nm, and $cf$ is a correction factor = (BRET/control/Rluc/control) with the negative control being the co-transfection of donor fusion proteins with YFP in the absence of the protein of interest. A positive interaction was assumed if an investigated protein pair results in a BRET ratio above the method-specific threshold of 0.094. The expression of a YFP-Rluc fusion protein as a control was included in every individual experiment. For evaluation of titration experiments, an bioinformatics algorithm was applied (16). First, titration experiments performed at increasing acceptor-to-donor ratios, and constant total DNA amounts were analyzed using the Akaike’s method (GraphPad Prism) to distinguish non-linear regression (true positive interaction) and linear regression (false positive interaction). For the non-linear regression model, parameters were subsequently calculated by $Y = \frac{BRET_{\text{max}}(X/BRET_{50} + X)}{\text{Donor ratios}}$, where $BRET_{\text{max}}$ is the maximal BRET ratio, and $BRET_{50}$ is the acceptor-to-donor ratio required to reach half-maximal BRET. An interaction was assumed to be true positive if the preferred model was non-linear, with an $R^2 > 0.8$. Second, linear regression analysis was applied for experiments performed at a constant acceptor-to-donor ratio but increasing DNA amounts. An interaction was assumed to be direct at parameters of $1/slope < 10$ and $\gamma$ intercepts when $X = 0$ at $\gamma > 0.05$. For BRET experiments probing the influence of elastase on protein interactions, HEK293 co-transfected with candidate proteins at increasing acceptor-to-donor ratios were incubated with 5 $\mu$g/ml elastase for 60 min before BRET measurements.

Co-IP—Co-IP in isolated human neutrophils was performed using the Dynabead technology (Invitrogen) as described previously (17). The presenilin-1 (Abcam, ab15456 monoclonal) was used. Moreover, co-IP to confirm the interaction of CXCR1 and APH-1 was performed in COS-7 cells co-transfected with candidate proteins carrying C-terminal V5 tag or N-terminal FLAG tag. Lysates of COS-7 cells were incubated with mouse anti-FLAG antibody (1:1000 dilution; Agilent Technologies) and $\mu$Mac beads (1:20 dilution; Miltenyi Biotec). Pre-equilibrated $\mu$Mac columns were loaded with the lysate followed by washing and preincubated with LDS sample buffer (95 °C) for 5 min. Bound material was resuspended in 45 $\mu$l of LDS buffer, and proteins were separated by SDS-PAGE (4–12% Novex NuPAGE Bis-Tris gel system, Invitrogen) at a constant voltage of 200 V for 90 min. Proteins were blotted onto nitrocellulose membranes subsequent to SDS-PAGE. Blocked membranes were incubated for 1 h with the primary antibody, mouse anti-V5 (1:5,000 dilution; Invitrogen), or mouse anti-FLAG antibody (1:1,000 dilution; Agilent Technologies) as control and 1 h with the secondary, goat anti-mouse IgG HRP conjugated antibody (1:10,000 dilution; Santa Cruz Biotechnology). Blots were visualized with SuperSignal West Femto Substrate (Thermo Scientific), and chemiluminescence was monitored after a DIANA III imaging system. Background-corrected images were quantified by use of Aida Image Analyzer software v.4.04 (Raytest).

Table 1

| Inhibitors                        | Concentration | Source                     |
|----------------------------------|---------------|----------------------------|
| Cytochalasin B                   | 50 $\mu$g/ml | Sigma                      |
| Anti-PAR-2 (transferrin)         | 100 $\mu$g/ml| Gift from Prof. Hollenberg |
| Anti-TLR4                        | 20 $\mu$g/ml | LifeSpan BioSciences       |
| DAPT                             | 100 $\mu$M   | Sigma                      |
| PD 98059                         | 10 $\mu$M    | Calbiochem                 |
| PP2                              | 20 $\mu$M    | Calbiochem                 |
| SB 203580                        | 1 $\mu$M     | Tocris                     |
| SLIGRL-NH$_2$                    | 10 $\mu$M    | Tocris                     |
| LRGILS-NH$_2$                    | 1 $\mu$M     | Tocris                     |
| Staurosporin from Streptomyces sp. | 240 $\text{nM}$ | Sigma                    |
| TAPI-1                           | 50 $\mu$M    | Calbiochem                 |
| Trypsin from porcine pancreas    | 200 $\mu$M   | Sigma                      |
| Thrombin from human plasma       | 30 NIH       | Sigma                      |
| $\gamma$-Secretase inhibitor I   | 30 $\mu$M    | Calbiochem                 |
| Wortmann                         | 100 $\text{nm}$ | Sigma                 |

**Statistical Analysis**—Statistical significance of differences was determined by paired two-tailed Student’s $t$ test. In all tests differences were considered significant at $p < 0.05$.

**RESULTS**

Secretases and PAR-2 Are Involved in Elastase-triggered Loss of CXCR1—To dissect intracellular pathways involved in elastase-mediated cleavage of CXCR1, we applied an unbiased small molecule screen covering different classes of (i) proteases, including MMPs, ADAM/α-secretases, and γ-secretases, (ii) kinases, and (iii) putative elastase receptors and pretreated isolated neutrophils with these compounds/blocking antibodies before elastase stimulation (see Table 1 for details). Pretreatment of isolated neutrophils with single protease inhibitors in non-toxic but inhibitory concentrations (based on their IC$_{50}$ values) had no significant effect on elastase-triggered CXCR1 cleavage (Fig. 1A). However, when our screen was expanded to combined inhibition of ADAM17 and γ-secretase activity, elastase-mediated CXCR1 cleavage was largely prevented, whereas inhibition of ADAM17 and γ-secretase alone had no significant effect. This initial finding was corroborated by immunoblotting of the extracellular C-terminal CXCR1 moiety (Fig. 1B) and confocal laser scanning microscopy (Fig. 1C) and was pharmacologically confirmed by a second class of γ-secretase inhibitors that showed a similar result (Fig. 1A).

Because α- and γ-secretases are mainly active intracellularly, we hypothesized that exogenous elastase activates intracellular secretases through activation of a surface receptor on neutrophils. Because TLR4 and PAR-2 have been previously involved in elastase-mediated cell activation, we used TLR4 and PAR-2 blocking antibodies in these assays. These experiments showed that blocking PAR-2, but not blocking TLR4 (Fig. 1A), had a significant effect on elastase-mediated CXCR1 loss comparable to the effect observed by combined α- and γ-secretase inhibition. When viewed in combination, these results demonstrate that combined α- and γ-secretase activity and PAR-2 are involved in elastase-mediated disintegration of CXCR1 expression on human neutrophils.

Secretases and CXCR1 Co-localize and Physically Interact—Next, we asked whether secretase components are expressed by human neutrophils and colocalize with CXCR1 at the single cell level. Confocal microscopy, flow cytometry, and immunoblotting (Fig. 2) confirmed the presence of the presenilin-1, the key component of γ-secretase harboring protease activity (18), in
isolated primary human neutrophils. Presenilin-1 was mainly localized intracellularly and at perimembranous regions and partially co-localized with CXCR1 in neutrophils (Fig. 2, A and B). BRET further demonstrated physical interaction of CXCR1 with other core components of the γ-secretase complex, particularly APH-1. BRET ratios determined for interaction of CXCR1 with presenilin-1 and with PEN-2 did not exceed a method specific threshold of 0.094, and the BRET ratio for nicastrin was even lower (Fig. 2C). However, a BRET ratio above the threshold was observed for basigin, recently shown to act as a key regulator of γ-secretase function (19). Moreover, the α-secretase ADAM10 and PAR-2 interacted with CXCR1. The interaction of CXCR1 with CXCR2 served as a positive control, and the interaction with the peroxisomal protein PEX14 as a negative control.

Next we performed fine-mapping of the CXCR1/γ-secretase network analyzing APH-1, which displayed the highest BRET ratio, as well as presenilin-1 as the active component of the complex. BRET titration experiments revealed that CXCR1 interacted directly with APH-1, whereas the interaction with presenilin-1 was found to be indirect (Fig. 2, D and E). By contrast, CXCR2 only indirectly interacted with both APH-1 and presenilin-1 (data not shown). This finding pointed to a specific non-redundant and functional interaction of CXCR1 with the γ-secretase, whereas CXCR2 was linked to the complex indirectly by interaction with CXCR1. This is consistent with our previous observations that CXCR1 (4), but not CXCR2, is processed by elastase in the airways of individuals with CF, thereby disabling the bacteria-killing capacity of neutrophils and modulating disease activity. To investigate whether the interaction

FIGURE 1. Secretases and PAR-2 are involved in elastase-triggered loss of CXCR1. A, isolated human neutrophils were treated with purified human neutrophil elastase (10 μg/ml for 60 min at 37°C in HBSS) with and without pretreatment with inhibitors for the enzymes/pathways/receptors as indicated in the figure legend and described in detail in Table 1. Afterward, the surface expression of CXCR1 was quantified using flow cytometry and a specific antibody (BD Biosciences clone 5A12, IgG2b). The percentage loss of CXCR1 surface expression in mean fluorescence intensity is shown in relation to elastase (100%). *, p < 0.05 versus elastase-treated neutrophils. GSI, γ-secretase inhibitor I. B, immunoblotting against the C-terminal moiety of CXCR1 after elastase treatment (10 μg/ml for 60 min at 37°C in HBSS) with or without inhibition of secretases (for 60 min at 37°C; 100 μM DAPT, 50 μM TAPI-1, 30 μM γ-secretase inhibitor I. C, CXCR1 surface expression was visualized by confocal laser scanning microscopy after elastase treatment (10 μg/ml for 60 min at 37°C in HBSS) with or without inhibition of secretases (see B for details). Nuclear DNA was stained with DAPI.
of CXCR1 with APH-1 depends on a functional catalytically active γ-secretase complex, further BRET experiments were performed upon co-expression of wild-type presenilin-1 or the mutant D257A. The D257A side chain replacement allows for γ-secretase complex formation but impedes the activating endoproteolytic cleavage of presenilin-1 (Fig. 2F). No impact on the interaction of CXCR1 with APH-1 was observed (p = 0.28) in the presence of D257A, indicating that the APH-1-mediated recruitment of CXCR1 to the γ-secretase complex did not depend on secretase activity. Finally, the direct physical interaction between CXCR1 and APH-1 was confirmed by co-immunoprecipitation (Fig. 2G).

To explore the functional context and to gain insight into cellular pathways of proteins involved in the identified interactions, we investigated the cellular protein network of CXCR1 and the α-/γ-secretases in more depth. Based on a database research (STRING, BioGRID, HPRD), a network of first order interactions was constructed (Fig. 2H). These studies confirmed the notion that the chemokine receptors CXCR1 and CXCR2 interact with each other through receptor cross-desensitization, cross-regulation (20), and heterodimerization (21) and thereby establish shared interactions with their cognate chemokine ligands (CXCL1, CXCL2, CXCL5, PPBP, CXCL8). CXCR1 and PAR-2 were further shown to be linked to the protein interaction cluster of the γ-secretase complex (APH-1, NCSTN, PEN-2, PSEN1) by interaction with APH-1. In addition, CXCR1 established an interaction with the α-secretase ADAM10. Second-order interactions of CXCR1 and PAR-2 with the γ-secretase complex were further established through CXCR1-BSG-PSEN1 and PAR-2-TMED2-NCSTN. The latter sequence of interactions may point to a shared pathway of membrane trafficking for PAR-2 and the γ-secretase complex. TMED2 is involved in anterograde COPII vesicle-mediated trafficking of G-protein-coupled receptors and nicastrin (NCSTN) has been described to play a role in γ-secretase trafficking (22). The immunoglobulin family member basigin or CD147 (BSG) is known as a regulator of [γ-secretase activity as well as inducer of MMP and as a receptor for the chemotactic cyclophilins (23). Moreover, the expanded network (Fig. 3) visualized how CXCR1 and PAR-2 are linked by the γ-secretase complex to functional protein interaction clusters of cell cycle control and the cadherins/catenins (24). Taken together, these results support an association of CXCR1 and PAR-2 with the γ-secretase complex, which is mediated by APH-1 and may be regulated by basigin.

PAR-2 Is Stored in Distinct Membrane-communicating Intracellular Neutrophil Compartment—Our studies so far provided evidence that secretases, PAR-2, and CXCR1 colocalize and physically interact and indicate that this interaction is involved in regulation of CXCR1 expression. Although CXCR1 expression in neutrophils has been analyzed and defined in detail, the expression and subcellular localization characteristics of PAR-2 in human neutrophils are poorly understood. To address this issue, we employed a subcellular fractionation approach as described previously by us (10, 13) and analyzed PAR-2 protein expression in subcellular neutrophil compartments. These studies provided evidence that PAR-2 is present in subcellular fractions representing the plasma membrane, secretory vesicles, and tertiary granules (Fig. 4A). Furthermore, BRET experiments revealed a protein interaction of PAR-2 with presenilin-1 and the γ-secretase component APH-1 (Fig. 4B). Consistent with the findings for CXCR1, co-expression of mutant presenilin-1-D257A did not influence the interaction of PAR-2 with APH-1 (p = 0.47) (Fig. 4C). This indicated that also PAR-2 is recruited to the γ-secretase complex by APH-1, a process independent of presenilin-1 activity.

Elastase Modulates γ-Secretase Components through PAR-2—These findings led us to speculate that free elastase stimulates intracellular γ-secretase activity and thereby orchestrates the disintegration of CXCR1 and the release of C-terminal CXCR1. To investigate the influence of elastase on the interactions of CXCR1 with the γ-secretase, co-immunoprecipitations in human neutrophils using Dynabead-based purification (17) of CXCR1 and probing with anti-presenilin-1 (Fig. 5A) were performed. We observed an increase of the co-IP signal when elastase was added to the isolated neutrophils (Fig. 5A). Furthermore, we observed that free elastase dose-dependently increased presenilin-1 protein expression in neutrophils (Fig. 5B). These data suggested that elastases may mobilize presenilin-1 in neutrophils. Therefore, we asked which elastase-downstream receptors on neutrophils mediate this effect. Based on our previous findings (Fig. 1A), we used a PAR-2 blocking anti-
body to define the pathway mobilizing presenilin-1 (Fig. 5B). The elastase-mediated effect was almost abolished when PAR-2 was blocked before elastase treatment using anti-PAR-2 antibodies or when PAR-2 was pre-activated with trypsin or a PAR-2 activating peptide before elastase treatment (Fig. 5, B and C). In contrast, PAR-1 pre-activation by using thrombin or control peptide pretreatment had no effect on elastase-mediated loss of CXCR1 (Fig. 5C), indicating a non-redundant role for PAR-2 in this process. Consistent with these findings in vitro, neutrophils isolated from the protease-dominated site of CF airway inflammation ex vivo, where elastase activities are abundant and CXCR1 cleavage occurs (4), showed increased presenilin-1 protein expression in CF airway neutrophils compared with peripheral blood neutrophils, which have no physical contact to free extracellular elastase activities (Fig. 5D). When viewed in combination, these studies demonstrate that elastase increased the expression of presenilin-1 in neutrophils through PAR-2 signaling and facilitated the physical interaction of presenilin-1 with CXCR1.

DISCUSSION

The surface expression of the G-protein-coupled chemokine receptor CXCR1 in neutrophils is regulated by proteases (4), but the underlying cellular network remained incompletely defined. Here we demonstrate, using a variety of different biochemical and immunological methods, that elastase activates a collaborative intracellular network involving PAR-2 and secretases, which regulates CXCR1 surface expression in human neutrophils.
FIGURE 4. PAR-2 is stored in membrane-communicating neutrophil compartments. A, resting neutrophils were gently disrupted using nitrogen cavitation method followed by four-layer gradient subcellular fractionation. Distribution profiles of myeloperoxidase (marker of azurophil granules), neutrophil gelatinase-associated lipocalin (marker of secondary/specific granules), MMP-9 (marker of tertiary granules), HSA (marker of secretory vesicles), and MICA/marker of plasma membranes (HLA), as measured by ELISA. The y axis represents an arbitrary scale with the ratio of PAR-2 band intensity to total protein. Shown is a Western blot analysis of neutrophil subcellular fractions and PAR-2 immunoblotting in isolated human neutrophils. PMN, whole neutrophils (cavitate); SV, secretory vesicles; PM, plasma membranes; 3°, tertiary granules; 2°, tertiary granules; 1°, primary granules. B, PAR-2 physically interacts with proteins of the γ-secretase (APH-1, presenilin-1). BRET ratios are given as the means ± S.D. of n = 2 replicates; and the red dotted line indicates the threshold for positive interactions of 0.094. C, interaction of PAR-2 with APH-1 does not depend on presenilin-1 function. Interaction of PAR-2 with APH-1 was determined upon co-expression of presenilin-1 or presenilin-1 carrying the loss-of-function mutation D257A. BRET ratios are given as the means ± S.D. of n = 2 replicates; the red dotted line indicates the threshold for positive interactions of 0.094.

FIGURE 5. Elastase modulates γ-secretase through PAR-2. A, co-IP of CXCR1 (C-terminal) in isolated human neutrophils using the Dynabead co-IP method (as described in detail under “Experimental Procedures”) and immunoblotting (IB) against N-terminal presenilin-1 with or without elastase (10 μg/ml for 60 min at 37 °C in HBSS). B, presenilin-1 surface expression on isolated human neutrophils was quantified using flow cytometry with three different elastase concentrations (1, 5, 10 μg/ml for 60 min at 37 °C in HBSS) with and without pretreatment with anti-PAR-2 blocking antibodies. C, PAR-2, but not PAR-1, preactivation protects from elastase-mediated loss of CXCR1 expression on neutrophils. Isolated human neutrophils were either untreated, treated with elastase only (10 μg/ml for 60 min at 37 °C in HBSS), or pretreated with thrombin (PAR-1 agonist, 30 NIH units), trypsin (PAR-2 agonist, 200 nM), a PAR-2 activating peptide (SLIGRL-NH2, 10 μM), or a control peptide (LRGILS-NH2, 10 μM) for 60 min and then treated with elastase (10 μg/ml for 60 min at 37 °C in HBSS). CXCR1 surface expression was assessed by flow cytometry. The percentage loss of CXCR1 surface expression in mean fluorescence intensity is shown in relation to elastase (100%). *, p < 0.05 versus elastase-treated neutrophils. D, presenilin-1 protein expression in neutrophils isolated from CF airway fluids or from corresponding peripheral blood neutrophils. Intracellular presenilin-1 protein expression was quantified by flow cytometry.
Collectively, the results of our study imply that elastase regulates CXCR1 surface expression in neutrophils in concert with PAR-2 (as elastase sensing receptor) and secretases (as intracellular downstream proteases). PAR-2 is canonically known to be activated by trypsin through cleavage of the receptor in the active site between amino acids Arg-36 and Ser-37. Serine proteases, such as elastase, cathepsin G, and proteinase 3, have been previously known to cleave PAR-2 C-terminal of the trypsin cleavage site, thereby disarming the trypsin-elicited G-protein-mediated calcium signaling through PAR-2 (25). However, a recent study challenged and extended this view by showing that neutrophil elastase can activate PAR-2 independent of disarming the tethered ligand-mediated mechanism by acting as a biased agonist (26). Based on our observation that elastase activates neutrophils and that PAR-2 blocking largely abrogated the effect of elastase on CXCR1 loss, we speculate that non-canonical PAR-2 activation through elastase as a biased agonist is critically involved in CXCR1 cleavage. Alternatively to PAR-2, TLR4 has been described to sense extracellular elastase (27). However, at least in our cellular modeling systems, only PAR-2 seemed to play a role in elastase-triggered CXCR1 modulation in neutrophils.

We further asked as to which PAR-2-downstream pathways are engaged in elastase-mediated CXCR1 disintegration. Based on previous studies on protease activities in human neutrophils (28), we focused on MMPs, as particularly MMP2, MMP8, MMP9, and MMP12 are expressed and active in neutrophils and have been shown to be activated through elastolytic activities (MMP2 and MMP9) (28). However, our studies did not provide any evidence for an involvement of MMPs in elastase-triggered CXCR1 modulation in human neutrophils. Likewise, inhibition of MAPK, src family kinases, PI3K, and PKC had no significant effects on elastase-mediated CXCR1 regulation. Unexpectedly, combined, but not single, inhibition of the α- and γ-secretase activity, best known from their role in Alzheimer disease, had a significant effect on elastase-triggered CXCR1 loss, pointing to a central role of these secretases in elastase/PAR2-mediated regulation of CXCR1 in neutrophils. Further experiments using flow cytometry, confocal microscopy, BRET, co-IP, and Western blotting confirmed these findings and provided evidence for a co-localization and physical interaction of CXCR1, PAR-2, and components of the α- and γ-secretase complex. The combined mode of action of α- and γ-secretases is reminiscent of the sequential protease involvement in processing of substrates of the γ-secretase, such as the amyloid precursor protein (29, 30). Besides amyloid precursor protein, secretases have been reported to play a role in cleaving and processing a variety of different proteins, including MHC class I proteins, in particular human leukocyte antigen A2 (31), the interleukin-1 receptor type 1 (IL-1R1) (32), leukosialin/CD43 (33), transmembrane-associated chemokines, the Notch receptor, and others (34). These processes probably occur close to the cellular membrane through juxtamembranous cleavage events as demonstrated previously for CD43 (33). Despite the fact that our BRET and co-IP experiments supported a physical interaction of secretases with PAR-2 and CXCR1, further studies are required to dissect the precise sequence of molecular events involved in this proteolytic cascade. An expanded interaction network of proteins analyzed in this study provided further evidence that the identified PAR-2/secretase network is itself regulated through cell-cycle control and cadherin/catenin-mediated cell-cell signaling pathways, an issue for further investigations.

Given this proposed network of proteases, protease receptors, secretases, and CXCR1, the question arises as to which relevance it has on neutrophilic inflammation in human disease conditions. CXCR1 is a central receptor for neutrophil recruitment and activation, supported by the importance of its cognate ligand CXCL8 in a variety of inflammatory diseases, prototypically in CF lung disease, where it represents an early disease biomarker (35, 36), but also in rheumatoid arthritis, sepsis, chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), diabetes, gout, and other disease conditions. Therapeutically, there are different strategies to target CXCR1 and/or CXCR2 (2) for both inflammatory diseases and cancer (1, 2, 37). To further pursue targeting CXCR1, however, a basic understanding of regulation and post-translational processing of this receptor is essential. We showed previously that elastase triggers the cleavage of CXCR1 and thereby prevents activation of the receptor and neutrophil effector functionalities (4). Here we extend this concept by showing that elastase, besides direct proteolytic effects, activates a complex intracellular network consisting of PAR-2 and secretases, which is, in turn, integrated in a more complex regulatory layer of proteases, chemokines, and kinases (Figs. 2 and 3). Beyond elastase and CXCR1, the complex nature of the identified protein-protein interaction network also indicates that the protease and chemokine system in neutrophils in general is physically and functionally interconnected in a more sophisticated manner than previously anticipated. Further dissecting and pharmacologically interfering with this network may pave the way for novel therapeutic strategies to specifically modulate neutrophilic inflammation at distinct intracellular checkpoints.

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