Cleavage of Signal Regulatory Protein α (SIRPα) Enhances Inflammatory Signaling*

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James D. Londino‡, Dexter Gulick‡, Jeffrey S. Isenberg‡§, and Rama K. Mallampalli‡¶

From the‡Acute Lung Injury Center of Excellence, Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine and§Department of Cell Biology and Physiology and Bioengineering, University of Pittsburgh, Pittsburgh, Pennsylvania 15213, ¶Medical Specialty Service Line, Veterans Affairs Pittsburgh Healthcare System, Pittsburgh, Pennsylvania 15213, ¶¶Vascular Medicine Institute, Starzl Transplantation Institute, Department of Pharmacology and Chemical Biology, Division of Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213

Signal regulatory protein α (SIRPα) is a membrane glycoprotein immunoreceptor abundant in cells of monocyte lineage. SIRPα ligation by a broadly expressed transmembrane protein, CD47, results in phosphorylation of the cytoplasmic immunoreceptor tyrosine-based inhibitory motifs, resulting in the inhibition of NF-κB signaling in macrophages. Here we observed that proteolysis of SIRPα during inflammation is regulated by a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), resulting in the generation of a membrane-associated cleavage fragment in both THP-1 monocytes and human lung epithelia. We mapped a charge-dependent putative cleavage site near the membrane-proximal domain necessary for ADAM10-mediated cleavage. In addition, a secondary proteolytic cleavage within the membrane-associated SIRPα fragment by γ-secretase was identified. Ectopic expression of a SIRPα mutant plasmid encoding a proteolytically resistant form in HeLa cells inhibited activation of the NF-κB pathway and suppressed STAT1 phosphorylation in response to TNFα to a greater extent than expression of wild-type SIRPα. Conversely, overexpression of plasmids encoding the proteolytically cleaved SIRPα fragments in cells resulted in enhanced STAT-1 and NF-κB pathway activation. Thus, the data suggest that combinational actions of ADAM10 and γ-secretase on SIRPα cleavage promote inflammatory signaling.

Inhibitory receptors including signal regulatory protein α (SIRPα),2 CD33, SIGLEC5 (sialic acid-binding immunoglobulin-like type 1 lectins), CD66a, PD-1, PILRa (paired immunoglobulin-like type 2 receptor α), CMRF35H, gp49B1, PECAM1, and others have been demonstrated to suppress the initiation of inflammatory signaling and contribute to the resolution of inflammation after infection (1, 2). SIRPα is a membrane glycoprotein immunoreceptor that is expressed mainly in myeloid and neuronal cells (3). Ligation of SIRPα results in phosphorylation of the cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which recruit and activate SH2 domain-containing phosphotyrosine phosphatases SHP-1 and SHP-2 (4). SIRPα signaling results in reduced macrophage migration and phagocytosis and inhibition of NF-κB signaling with reduction of release of NF-κB-dependent cytokines in macrophages (5, 6). Phosphorylation of SIRPα ITIM domains also enhances JAK/STAT activation and NADPH oxidase expression and activity (7). Thus, SIRPα serves as an important modulator of the host adaptive immune response.

Proteolysis and release of the SIRPα NH2-terminal domain has been demonstrated in primary cultured neurons, melanoma cells, and macrophage cell lines. Cleavage of murine SIRPα through an MMP inhibitor-sensitive pathway was first observed in cultured cells engineered to express both SIRPα and an active form of Ras (8). In these cells, blocking SIRPα proteolysis resulted in inhibited cell migration, cell spreading, and cytoskeletal reorganization. In addition, SIRPα was shown to regulate synaptic activity through activation of MMP inhibitor-sensitive proteolysis in primary murine neurons wherein the NH2-terminal domain of postsynaptic SIRPα promoted the maturation of the presynaptic terminal through a CD47-dependent mechanism (9). However, the identification, characterization, and regulation of SIRPα cleavage products in inflammatory signaling have not been investigated in myeloid cells. In addition to SIRPα expression in brain tissue and in myeloid cells, SIRPα is also detected at lower levels in heart, placenta, lung, testis, ovary, colon, liver, small intestine, prostate, spleen, kidney, skeletal muscle, and pancreas. The role of SIRPα in the pulmonary epithelium has not been explored and may also play a role in the resolution of inflammatory lung injury.

In these studies, we describe the role of proteolysis of SIRPα in response to proinflammatory stimuli. We determined that the inflammatory mediators lipopolysaccharide (LPS) and...
tumor necrosis factor-α (TNFα) increase SIRPα proteolysis in a THP-1 human monocyte cell line and in human lung epithelial cells. We mapped a putative ADAM10 cleavage region within the extracellular juxtamembrane region and demonstrate a requirement for this enzyme for proteolysis of human SIRPα. Finally, we discovered that after primary proteolysis the SIRPα membrane-associated fragment is rapidly cleaved by γ-secretase where it is released into the cytosol. Cellular expression of plasmids encoding these SIRPα families was used to modulate cellular inflammatory signaling, thus providing a physiological role for SIRPα sequential proteolysis in human cells.

Materials and Methods

Cells—THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS). HeLa cells were cultured with Eagle’s minimal essential medium containing 10% FBS and antibiotics. RAW264.7 cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS and antibiotics. BEAS-2B cells were maintained with HITES (hydrocortisone, insulin, transferrin, estradiol, and selenium added to RPMI 1640 medium) supplemented with 10% FBS.

Chemicals Used—The following reagents were purchased and used at the following concentrations unless otherwise indicated: bortezomib, 10 µM (Santa Cruz Biotechnology); Compound 2, 50 µM (EMD Millipore); cycloheximide (CHX), 10 µg/ml (Fisher); DAPT, 10 µM (Santa Cruz Biotechnology); GZ254032X (Toocris); GM6001, 50 µM (Enzo Life Sciences); lactacystin, 10 µM (Cayman Chemical); leupeptin, 50 µM (Sigma); LP5, 1 µg/ml (Sigma); MMP2/9 inhibitor I, 25 µM (Santa Cruz Biotechnology); MG132, 20 µM (Calbiochem); PD 150606 (Santa Cruz Biotechnology); PMA, 100 nM (Sigma); PMSF (Sigma); and recombinant human TNFα, 10 ng/ml (eBioscience).

Plasmids, Cloning, and Transfection—Human SIRPα (hSIRPα; NM_007547.2) (Sino) were cloned into a pcDNA3.1/V5-HisTOPO cloning kit (Invitrogen). FLAG domains were inserted into indicated regions near the SIRPα extracellular juxtamembrane region using non-overlapping primers using a QuikChange site-directed mutagenesis kit (Agilent). Point mutants were also generated using the QuikChange site-directed mutagenesis kit (Agilent). SIRPα and the SIRPα COOH-terminal domain (amino acids 359–504 of hSIRPα) (OriGene) and murine SIRPα (mSIRPα; NM_007547.2) (Sino) were cloned into a pcDNA3.1 vector using a pcDNA3.1/V5-HisTOPO cloning kit (Invitrogen). SIRPα cytoplasmic + membrane (SIRPα-c-m) and SIRPα cytoplasmic fragment (SIRPα-cyto-C) were generated using pcDNA3.1/V5-HisTOPO cloning kits (Life Technologies). A putative SIRPα-c-m ORF was generated based on our mapping and site-directed mutagenesis studies and contains amino acids 339–504 of hSIRPα. The SIRPα-cyto-C contains the entire COOH-terminal domain of SIRPα excluding the transmembrane domain (amino acids 398–504 of hSIRPα). Plasmids were transfected into cells using XtremeGene transfection reagent (Roche Applied Science) or Turbofect transfection reagent (Life Technologies) according to the manufacturer’s protocols.

RNAi—DsiRNA was obtained from Integrated DNA Technologies, Inc. oligonucleotides. These include: ADAM10 DsiRNA 2: sense, 5’-CCACUAAAGAU- GAGUAUUUUCUTA-3’; antisense, 5’-UAAGCAAAUAU- ACUCUUUAUUGGUU-3’; and ADAM10 DsiRNA 3: sense, 5’-GCAGAAUCUGACUGUUTG-3’; antisense, 5’-CCCAAAGUAGUCCUAUCUCUCGCUC-3’. THP-1 and HeLa cells were transfected with 10–30 nM DsiRNA using GeneMute siRNA transfection reagent (SignaGen) according to the manufacturer’s instructions.

Immunoblotting—Cell lysis and immunoblotting were performed as described previously (10). Briefly, cells were lysed in PBS supplemented with protease inhibitors (Thermo Scientific), and lysates were sonicated and then centrifuged at 500 × g to remove debris. An aliquot of sample containing total cellular protein was frozen at −80 °C. Subcellular fractionation, cells were also centrifuged at 16,000 × g to pellet membranes. The supernatant containing the cytoplasmic protein was removed and stored at −80 °C. The resultant crude membrane pellet was resuspended in radioimmunoprecipitation assay buffer supplemented with protease inhibitors, briefly sonicated, and stored at −80 °C. Total cellular, soluble, and membrane proteins were loaded on SDS-polyacrylamide gels and processed for immunoblotting using the following antibodies SIRPα COOH-terminal antibody targeting amino acids 487–503 (566310, Millipore), SIRPα COOH-terminal antibody targeting residues surrounding Pro-413 (D613M, Cell Signaling Technology), V5 (Life Technologies), FLAG M2 antibody (Sigma), GAPDH (Sigma), STAT1 (7649P, Cell Signaling Technology), pSTAT1 (7649P, Cell Signaling Technology), ADAM10 (AB19026, EMD Millipore), and β-actin mouse monoclonal antibody (Sigma). Immunoblots were exposed to SuperSignal West Femto chemiluminescent substrate (Thermo Scientific). Extracellular proteins were isolated from culture medium that was concentrated using an Amicon Ultra-4 centrifugal filter unit with Ultracel-30 membrane (EMD Millipore) before processing for immunoblotting.

Fluorescent Immunostaining—HeLa cells were inoculated into glass-bottomed 35-mm plates and transiently transfected with the indicated plasmids for 24 h. Cells were treated as indicated, washed with cold PBS twice, and fixed with 4% paraformaldehyde for 10 min prior to incubation of the fixed cells with staining solution (0.1% Triton X-100 in PBS with 1% goat serum) for 30 min. The cells were then probed with a SIRPα COOH-terminal monoclonal antibody (Cell Signaling Technology) (1:300) or V5 antibody (Invitrogen) (1:300) in staining solution overnight. Plates were washed three times and incubated with fluorescence-conjugated goat anti-rabbit secondary antibody (1:500) for 1 h. Plates were then washed three times for 10 min. DAPI was then added (1:5000) for 5 min. Images were acquired using a combination of laser-scanning microscope systems (Nikon A1, Nikon, Melville, NY), and the results were analyzed using Nikon NIS-Elements software.

Bacterial Infection—Pseudomonas aeruginosa (PA103) were cultured as described previously (11). Briefly, inocula were freshly prepared prior to experiments from frozen stocks of PA103. Overnight plate cultures were then inoculated in tryptic soy broth supplemented with 1% glycerol and 100 mM sodium glutamate and grown by rotary shaking at 37 °C to log phase. Cells were infected with PA103 as described.
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**Results**

**SIRPα Is Cleaved by a Matrix Metalloproteinase in Response to Inflammatory Mediators**—To determine the half-life of SIRPα, we incubated the human monocytic cell line THP-1 with the protein biosynthesis inhibitor CHX and measured protein lifespan; a $t_{1/2}$ of ~2 h was indicated (Fig. 1a). Although we observed a decrease in SIRPα protein in the presence of CHX, this turnover did not appear to be affected by either a lysosomal inhibitor, leupeptin, or a proteasomal inhibitor, MG132 (Fig. 1a), suggesting that the full-length protein is processed by an alternative pathway. However, in these studies, we consistently observed a decrease in SIRPα protein in human macrophages (RAW264.7) treated with LPS, TNFα, or a combination of both (Fig. 1b), (8). Because proteolysis of murine SIRPα is also enhanced by PMA stimulation (8), we incubated THP-1 with PMA and measured the degradation of full-length murine SIRPα and the production of SIRPα + m. Degradation of full-length SIRPα and the appearance of SIRPα + m was dose-dependently increased by PMA (Fig. 1c).

SIRPα proteolysis in RAW264.7 macrophages resulted in the production of a similar MG132-sensitive fragment (Fig. 1f). To determine whether SIRPα proteolysis plays a biologic role in macrophage signaling, we treated cells with the proinflammatory mediator LPS. In a time course analysis of SIRPα proteolysis in THP-1 cells, LPS treatment led to a decrease in abundance of full-length SIRPα at ~1 h post-treatment, an effect that was inhibited by treatment of cells with a broad spectrum MMP inhibitor, Compound 2 (12) (Fig. 1f). Although it is reasonable to predict from the LPS-induced disappearance of full-length protein that SIRPα + m was being generated, this fragment was not readily detected again unless stabilized by MG132 treatment (Fig. 2a, lower blots). In comparative analysis of proteolysis of SIRPα, LPS and TNFα enhanced the accumulation of SIRPα + m in THP-1 cells to a similar extent as actions of PMA with appearance of a fragment identical to the PMA-generated product (Fig. 2b). With each stimulus, we were unable to observe the production of SIRPα + m without inclusion of MG132 in the culture medium to stabilize the fragment.

**SIRPα Proteolysis Is Charge-dependent**—To identify the site of proteolytic cleavage in human SIRPα, we generated a series of proteolytic cleavage in human SIRPα, we generated a series...
of FLAG-tagged SIRPα mutants where we substituted amino acids in the full-length construct NH2-terminally adjacent to the transmembrane domain with the sequence DYDDDDK (Fig. 3a). This sequence of amino acids, termed FLAG tag, is recognized by commercially available antibodies. We then measured the association of the FLAG domain using a specific antibody that will either associate with the secreted NH2-terminal domain or the membrane-bound COOH-terminal domain depending on the site of proteolysis. If the cleavage site is upstream of the FLAG domain resulting in the FLAG epitope that is attached to the SIRPc+m domain, we should observe a reactive band at the size of the SIRPc+m fragment when probing with either the FLAG antibody or the SIRPα COOH-terminal antibody generated against residues 487–503 (Fig. 3b, left). Alternatively, if the site of cleavage is downstream of the inserted FLAG domain, the FLAG epitope will be associated with the secreted NH2-terminal domain without reactivity with the FLAG antibody when probing cell lysates. However, in this latter scenario, a FLAG-reactive band should be detected in the medium at the predicted size of the SIRPα cleaved fragment (Fig. 3b, right). Because HeLa cells are readily transfectable, we first evaluated whether overexpressed wild-type (WT) SIRPα was subject to proteolysis by analyzing production of the SIRPc+m fragment. As observed in THP-1, PMA enhanced the production of the SIRPc+m. MG132 also increased the abundance of SIRPc+m (Fig. 3c). HeLa cells were then transfected with the various FLAG-containing SIRPα plasmid constructs, and cells were then stimulated with PMA to activate proteolysis and MG132 to preserve the SIRPc+m fragment, respectively. Cell lysates were then processed for immunoblotting using a COOH-terminal SIRPα antibody or an anti-FLAG antibody. We observed that expression of only the FLAG site 1 construct in cells resulted in a FLAG-reactive band in the SIRPc+m fragment (Fig. 3d). Hence, these results suggest that SIRPα cleavage occurs between FLAG sites 1 and 2 (Fig. 3, a and d). Due to the lack of an acceptable antibody against the hSIRPα NH2-terminal domain, we chose to characterize the proteolysis and release of the SIRPα NH2-terminal domain using our FLAG insertional mutants. After determining that FLAG site 3 was not associated with the SIRPc+m domain and was cleaved normally, we transfected HeLa cells with a plasmid encoding FLAG site 3 and examined proteolysis. PMA treatment of cells increased the appearance of the SIRPc+m fragment in cell lysates (Fig. 3e). We then confirmed the release of the SIRPα NH2 terminus into the medium by probing for the FLAG domain in the supernatants. PMA was sufficient to trigger the release of the SIRPα NH2-terminal domain into the culture medium, an effect abrogated by MMP inhibition (Fig. 3e). Although MG132 significantly increased the stability of SIRPc+m, it had little effect on the abundance of the extracellular fragment, suggesting that proteolysis itself is not altered by MG132. Thus, these data suggest that SIRPα is indeed clipped, resulting in the generation of both an extracellular released NH2-terminal fragment and a membrane-associated cleavage product. Having determined that the approximate site of proteolysis was between FLAG sites 1 and 2, we introduced a FLAG tag between these two regions (termed SIRPα FLAG site 1.5). Compared with expression of the FLAG site 1 and 2 constructs, cellular expression of the SIRPα FLAG site 1.5 variant dramatically inhibited both the appearance of SIRPc+m and the extracellular release of the SIRPα NH2-terminal domain (Fig. 3f). Because alterations near the transmembrane domain could potentially disrupt protein folding or trafficking, we performed immunofluorescence imaging to characterize the membrane localization of FLAG site 1.5 after cellular expression. In HeLa cells, both the WT SIRPα and the FLAG site 1.5 mutant appear to associate with the plasma membrane to a similar extent (Fig. 3g). To further ascertain the specific molecular attack sites for SIRPα cleavage, we generated a series of point mutations within the FLAG domain of SIRPα based on predictions of which motifs may be important. Substitution of Ala at residue 359 did not alter cutting, but substitution of Lys at residue 359 enhanced proteolysis. Substitution of Ser-358 and Thr-360 with negatively charged residues resulted in reduced SIRPα proteolysis (data not shown). Because of these results and because the FLAG tags are enriched with negatively charged residues, we hypothesized that the inhibition of proteolysis by the FLAG site 1.5 domain was charge-mediated. Therefore, we generated additional FLAG mutant constructs retaining only the negatively charged Asp residues (Fig. 4a). FLAG site 1.5, FLAG-D1, and FLAG-D2 constructs effectively blocked SIRPα proteolysis compared with the WT FLAG domain (Fig. 4b). In contrast, replacing the same residues with a series of basic positively charged residues (Arg) (FLAG-R) drastically enhanced proteol-
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Identification of a juxtamembrane region of SIRPα proteolysis. a, schematic of FLAG domains replacing amino acid residues in the SIRPα extracellular membrane-proximal region. Trans, transmembrane domain; Wt, wild-type SIRPα. b, diagram depicting the association of the FLAG domain with both SIRPc-m and the extracellularly released NH2-terminal fragment. c, HeLa cells were transfected with a WT SIRPα-expressing plasmid. At 24 h post-transfection, medium was replaced with serum-free medium, and cells were incubated with vehicle, PMA, or MG132 (MG) for 6 h as indicated. d, HeLa cells were transfected with the various FLAG-containing SIRPα constructs shown in a. At 24 h post-transfection, the cells were incubated with PMA and MG132 for 6 h. SIRPα proteolysis was examined by immunoblotting using a SIRPα COOH-terminal antibody (top and third panels) or an antibody against the FLAG domain (second and bottom panels). FL, full-length SIRPα. e, HeLa cells were transfected with a plasmid encoding the FLAG site 3 construct representing release of the NH2-terminal domain (a). At 24 h post-transfection, medium was replaced with serum-free medium, and cells were incubated with PMA and MG132 for 4 h and examined by immunoblotting using a SIRPα COOH-terminal antibody (second and third panels) and an antibody against the FLAG domain (top panel) to detect the cleavage and release of SIRPα. f, HeLa cells were transfected with various FLAG constructs as indicated and treated as described in d. Both cell lysates and concentrated cell medium were analyzed by immunoblotting using a SIRPα COOH-terminal antibody (top and fourth panels) or a FLAG targeted antibody (second, third, and fifth panels). g, HeLa cells were transfected with plasmids expressing WT SIRPα or a FLAG site 1.5 SIRPα mutant and examined for expression at 24 h post-transfection. Green, SIRPα; blue, DAPI. Scale bar, 5 μm. Each panel is representative of two to three experiments. Comp2, Compound 2.

ysis, resulting in enhanced accumulation of the SIRPc-m product (Fig. 4b). Because the site of cleavage we identified was flanked by two negatively charged Glu residues, we hypothesized that a centrally placed negative residue should significantly limit SIRPα cleavage. Mutagenesis of Asn-359 to a negatively charged residue (Asp or Glu) greatly reduced SIRPα proteolysis under baseline and PMA-induced conditions (Fig. 4, c and d). In contrast, replacement of Asn-359 with a positively charged residue (Lys or Arg) enhanced proteolysis reflected primarily by loss of the parent SIRPα (Fig. 4, c and d). Mutation of this residue to an uncharged amino acid had no major effect on proteolysis (Fig. 4, c and d).

To further examine charge-dependent regulation of SIRPα, we analyzed proteolysis of mSIRPα to determine whether similar residues were important for cleavage. We transfected HeLa cells with plasmid encoding mSIRPα and measured PMA-mediated proteolysis. Like hSIRPα, mSIRPα was cleaved in response to PMA treatment, resulting in the production of a COOH-terminal fragment (Fig. 4e). In these studies, this fragment was subsequently determined to be cleaved by γ-secretase (see Fig. 6), and thus both MG132 and DAPT were used interchangeably as γ-secretase inhibitors to preserve the cleaved fragment. Murine SIRPα contains a Gln residue (Gln-361) in a similar location to the juxtamembrane Asn residue (Asn-359) in hSIRPα. Because mutation of the amide-containing amino acid Asn in hSIRPα drastically altered proteolysis, we made a series of point mutants near the Gln residue in mSIRPα (Asn-359) in hSIRPα. Because mutation of the amide-containing amino acid Asn in hSIRPα drastically altered proteolysis, we made a series of point mutants near the Gln residue in mSIRPα, speculating that this residue may also be important for proteolysis (Fig. 4a). Consistent with our observations in hSIRPα, the introduction of negatively charged residues in place of Gln significantly inhibited mSIRPα proteolysis, whereas the substitution of a neutral amino acid, Ala, had no effect. The introduc-
tion of three Asp residues was even more efficient at inhibiting proteolysis, suggesting a charge-dependent cutting mechanism. These results suggest that charge-mediated proteolysis is conserved across species despite major differences in the juxtamembrane domain sequence (Fig. 4, a and e).

**ADAM10 Is Responsible for SIRPα Proteolysis**—Although cleavage of SIRPα is inhibited by broad spectrum MMP inhibitors, the specific protease(s) responsible has not been identified. Ohnishi et al. (8) suggested that proteolysis of murine SIRPα might be MMP9-dependent. We therefore interrogated the role of MMP9 in THP-1 using a specific MMP2/9 inhibitor. Although the broad spectrum MMP inhibitor GM6001 (GM) blocked SIRPα degradation, the MMP2/9 inhibitor had no effect (Fig. 5a). PMSF, a serine protease inhibitor, also had no effect on proteolysis. We then tested whether Compound 2, an MMP8 inhibitor, could block proteolysis because it had been shown previously to inhibit the disappearance of membrane SIRPα in apoptotic neutrophils (13). Although Compound 2 was effective in inhibiting SIRPα cleavage, this antagonist was subsequently shown to be promiscuous, inhibiting many MMP and ADAM proteases (Fig. 5b) (12). Given the membrane-proximal site and rapid onset of proteolysis, we hypothesized that the membrane-bound ADAM protease family member was likely responsible for SIRPα shedding (14, 15). The specific ADAM10 inhibitor GI254023X (GI) blocked LPS-stimulated shedding by ~60% at 0.1 μM and completely blocked cleavage of SIRPα at 1 μM (Fig. 5b). To further examine the effects of the GI inhibitor on proteolysis, we overexpressed hSIRP and mSIRP in HeLa cells pretreated with GI and measured proteolysis in response to PMA. GI, like the broad spectrum MMP inhibitor GM, significantly reduced hSIRP and mSIRP proteolysis (Fig. 5c). Interestingly, GI was equally effective at inhibiting proteolysis in the hSIRPα mutants N359Q and N359A and in the mSIRPα mutants Q361N and Q361A, suggesting that these residues were not essential for ADAM10 specificity (data not shown). To directly examine whether endogenous ADAM10 was the protease responsible for SIRPα degradation, we transfected THP-1 cells with DsiRNA targeting ADAM10. ADAM10 knockdown appeared sufficient to prevent LPS-induced cutting of SIRPα as evidenced by increased full-length SIRPα, suggesting protection from proteolysis (Fig. 5d). To more thoroughly characterize the role of ADAM10 in SIRPα degradation, we transfected HeLa cells with DsiRNA against ADAM10; this was followed by transfection of the SIRPα FLAG site 3 mutant (Fig. 5e) that was stabilized by MG132. Knockdown of ADAM10 decreased both the release of the NH2-terminal SIRPα fragment and the appearance of SIRPα+m (Fig. 5e).

**The ADAM10 Cleaved Fragment Is Processed by γ-Secretase**—Thus far, the data support that ADAM10 proteolysis of SIRPα results in the appearance of a transient, membrane-associated cleaved fragment (SIRPα+m) that was stabilized by MG132. However, when we further examined the stability of SIRPα+m in the presence of MG132, we realized that this effect was only observed at higher concentrations, indicative of a proteasome-independent effect of the inhibitor given its nonspecificity.
These results were confirmed by the presence of a predicted proteasome inhibitor bortezomib that did not stabilize SIRPc+m (Fig. 6a). MG132 has also been shown to inhibit calpains, cathepsins, and \( \gamma \)-secretase in the micromolar range (16). We tested the ability of the calpain inhibitor PD150606 to prevent degradation of the SIRPc+m and observed that it had no effect on preserving the stability of the SIRPc+m fragment (Fig. 6b). ADAM10 proteolysis of substrates often leads to secondary cleavage by \( \gamma \)-secretase (17). Because MG132 is known to inhibit \( \gamma \)-secretase activity, we tested whether the \( \gamma \)-secretase inhibitor DAPT could prevent the disappearance of SIRPc+m. In contrast to calpain inhibitors, DAPT prevented the disappearance of SIRPc+m (Fig. 6c).

To further understand cleavage of SIRP\( \alpha \), we sought to characterize the fragment produced after \( \gamma \)-secretase-mediated SIRPc+m proteolysis. However, cytosolic fragments that are produced after \( \gamma \)-secretase cleavage are very unstable under native conditions (18–20). Because we also did not detect a \( \gamma \)-secretase-cleaved SIRP\( \alpha \) product, we sought to enhance the stabilization of the cytoplasmic SIRP\( \alpha \) fragment with the proteasome inhibitor lactacystin. HeLa cells were transfected with WT SIRP\( \alpha \) followed by treatment with lactacystin. The proteasome inhibitors bortezomib and lactacystin were used interchangeably throughout the experiments as these agents exerted similar effects. Proteasomal inhibition resulted in the accumulation of a small, low intensity band of the approximate predicted size of SIRPcyto-C (Fig. 6d; * indicates the SIRPcyto-C fragment). Furthermore, we did not observe the appearance of this fragment after treatment with DAPT, suggesting that \( \gamma \)-secretase proteolysis is necessary for the release of the SIRPcyto-C. To better visualize the proteolysis of SIRPc+m by \( \gamma \)-secretase, we cloned a construct resembling the ADAM10-cleaved SIRP\( \alpha \) fragment (pSIRPc+m). pSIRPc+m was associated with the membrane fraction in HeLa cells just as the WT SIRP\( \alpha \) cleavage product did (Fig. 6e). pSIRPc+m was subject to spontaneous proteolysis by \( \gamma \)-secretase, leading to the release of the cytoplasmic domain (Fig. 6f). To determine whether SIRPcyto-C is produced by endogenously expressed SIRP\( \alpha \), we treated THP-1 cells with the proteasomal inhibitors lactacystin (data not shown) and bortezomib. Both inhibitors led to the production of a DAPT-sensitive cleavage fragment of the predicted size (Fig. 6g). We also confirmed the generation of SIRPcyto-C in RAW267.3 murine macrophages (Fig. 6h). These data indicate that SIRP\( \alpha \) proteolysis results in the secondary cleavage by \( \gamma \)-secretase and the generation of a cytosolic COOH-terminal fragment.

**SIRP\( \alpha \) Is Expressed and Proteolytically Regulated in the Lung Epithelium**—Although SIRP\( \alpha \) signaling has been well described in myeloid cells and the brain, SIRP\( \alpha \) activity has been detected in other cell types and tissues. Because the lung epithelium is uniquely exposed to airborne stimuli and pathogens that trigger inflammatory signaling, we examined SIRP\( \alpha \) expression in...
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FIGURE 6. SIRPc+m undergoes proteolysis by γ-secretase. **a**, THP-1 cells were treated with PMA to stimulate proteolysis and then incubated with the indicated concentrations of the proteasome inhibitors MG132 (MG) or bortezomib (BTZ) at 30 min post-treatment. Cells were harvested at 4 h post-PMA treatment for immunoblotting using the indicated antibodies. **b**, THP-1 cells were treated with LPS for 30 min and then treated with the calpain inhibitor PD150606 (PD) at the indicated concentrations, and cells were harvested 4 h post-LPS treatment for immunoblotting as indicated. **c**, THP-1 cells were treated with LPS for 30 min and then DAPT at the indicated concentrations and harvested 4 h post-LPS treatment. **d**, HeLa cells were transfected with a WT SIRPα-expressing plasmid. At 24 h post-transfection, cells were incubated with or without DAPT, lactacystin (Lact), or vehicle control. At 30 min post-DAPT and lactacystin treatment, cells were incubated with PMA or vehicle for 4 h. **e** represents a γ-secretase-sensitive cleavage product termed SIRPcyto-C. **f**, SIRPα mutant plasmids were expressed in HeLa cells, and whole cell lysates (WCL) or cellular fractions were analyzed for proteolysis by immunoblotting 24 h after transfection. The SIRPc+m fragment encodes the putative ADAM10-cleaved SIRPα fragment (amino acids 359–504), whereas SIRPcyto-C encodes the potential γ-secretase-cleaved COOH-terminal domain of SIRPα (amino acids 398–504) followed by a V5 tag. **g**, HeLa cells were transfected with a plasmid expressing the putative SIRPc+m domain. At 24 h post-transfection, cells were incubated with vehicle, DAPT, or lactacystin. Cells were harvested 4 h post-DAPT/lactacystin treatment. **h**, THP-1 cells were pretreated with bortezomib and/or DAPT. 30 min later, cells were treated with PMA for 4 h. RAW264.7 macrophages were pretreated with lactacystin for 30 min prior to incubation with PMA with or without DAPT. Each panel is representative of two to three experiments. **Mem**, membrane; **Cyto**, cytosol; **Sig**, signal sequence; **c+m**, cytoplasmic + membrane domain.

24 h post-transfection, cells were incubated with vehicle, DAPT, or lactacystin. Cells were harvested 4 h post-DAPT/lactacystin treatment. **g**, THP-1 cells were pretreated with bortezomib and/or DAPT. 30 min later, cells were treated with PMA for 4 h. RAW264.7 macrophages were pretreated with lactacystin for 30 min prior to incubation with PMA with or without DAPT. Each panel is representative of two to three experiments. **Mem**, membrane; **Cyto**, cytosol; **Sig**, signal sequence; **c+m**, cytoplasmic + membrane domain.

These findings confirm that SIRPα is both expressed and subject to ADAM10- and γ-secretase-mediated proteolysis in the lung and that this process is increased in response to inflammatory pathogens.

**Proteolysis of SIRPα Enhances Inflammatory Signaling**—To determine whether proteolysis modifies SIRPα signaling, we measured inflammatory signaling in HeLa cells transfected with SIRPα mutant constructs. The IkB kinase complex (IKK) is an upstream NF-κB regulator. Phosphorylated IKKα and IKKβ result in dissociation of the inhibitory IkBa protein from NF-κB, which is then transported into the nucleus where it transcriptionally activates gene targets (21). HeLa cells were minimally responsive to LPS; however, treatment of HeLa cells with TNFα led to a transient induction of pIKKα/β phospho-
FIGURE 7. SIRPα is expressed and proteolytically regulated in human lung epithelia. a and b, lysates from normal human bronchial epithelium (NHBE) derived from three separate subjects (1, 2, and 3), the alveolar type II-like cell line A549, and the human bronchiolar epithelial cell line BEAS-2B were analyzed for SIRPα expression by immunoblotting. In b, human bronchial epithelial cells were probed for SIRPα-m. Patient sample 3 exhibited a cleaved fragment at the same mobility on gels as SIRPα-m when probed with a SIRPα COOH-terminal antibody. c, BEAS-2B cells were pretreated with GM or vehicle. At 30 min post-GM treatment, DAPT was added, and 30 min post-DAPT treatment, cells were incubated with PMA or vehicle for 4 h. d, BEAS-2B cells were incubated with vehicle, DAPT, or bortezomib (BZ) as indicated. At 30 min post-DAPT and bortezomib treatment, cells were incubated with PMA or vehicle for 4 h. e, BEAS-2B cells were incubated with DAPT for 30 min prior to incubation with PA103 (multiplicity of infection, 10) for 8 h following incubation with PA103. Densitometry of the ratio of cleaved fragment versus full-length SIRPα is shown on the right (control (Con), n = 4; GI, n = 3; control (Con) PA103, n = 5; GI PA103, n = 5; four individual experiments; mean ± S.D. (error bars), * p < 0.05 versus uninfected control; #, p < 0.05 versus control PA103). h, BEAS-2B were transfected with control or ADAM10 (A10)-targeted DsiRNA for 48 h followed by incubation with PA103 (multiplicity of infection, 10). Densitometry of the ratio of cleaved fragment versus full-length SIRPα (DsiRNA control (DsiCon), n = 3; ADAM10-targeted DsiRNA (DsiA10), n = 3; three individual experiments; mean ± S.D. (error bars)). Densitometry is shown on the right. * p < 0.05 versus DsiRNA control. The inset shows levels of ADAM10 protein after DsiRNA transfection.

Discussion

The new contributions of this study include (i) that SIRPα proteolysis and generation of degradation products enhance...


inflammatory signaling, (ii) the identification of ADAM10 as the metalloprotease responsible for human SIRPα proteolysis, (iii) that cleaved SIRPα is targeted for secondary proteolysis by γ-secretase releasing a COOH-terminal fragment, and (iv) that SIRPα processing in this manner is widespread including in primary human lung epithelia, suggesting that SIRPα proteolysis may be biologically important. SIRPα proteolysis may modify numerous diseases where SIRPα signaling has been implicated including cancer (26), renal ischemia reperfusion injury (27), stroke (28), Crohn disease, (29), and allergic airway inflammation (30).

Based on the central inhibitory role of SIRPα in the inflammatory response in macrophages, we investigated SIRPα protein turnover in murine cells. Although we did not observe proteosomal or lysosomal degradation of the full-length protein, we did detect the appearance of a small fragment that resembled a previously described, MMP inhibitor-sensitive degradation product (Fig. 1b) (8). To determine the pathobiological role of inflammatory signaling linked to degradation of SIRPα, we treated THP-1 cells with known proinflammatory stimuli, TNFα and LPS. Kong et al. (5) suggested that SIRPα disappearance in RAW264.7 macrophages in response to LPS was mediated by decreased transcription and lysosomal degradation of the immunoreceptor. In contrast, we observed that the LPS-mediated disappearance of SIRPα could be completely prevented by broad spectrum MMP inhibitors.

Although the proteolytic degradation of SIRPα had been described previously, the only evidence of SIRPα cleavage by specific proteases comes from in vitro degradation of purified murine SIRPα with recombinant MMP1 and MMP9 (8). However, there is no evidence that MMP9 cleavage actually occurs in intact cells. Our experiments suggest that MMP9 is not responsible for SIRPα degradation in either THP-1 (Fig. 4a), HeLa cells transfected with SIRPα (data not shown), or BEAS-2B because a specific MMP2/9 inhibitor did not prevent proteolysis. MMP8 was also characterized as a protease responsible for SIRPα proteolysis in primary neutrophils (13). However, this may not be the case because the inhibitor used in those experiments was shown to be promiscuous, inhibiting many MMPs and ADAM proteases (12, 31). Using specific inhibitors and RNAi, we determined that ADAM10 was involved in SIRPα proteolysis in epithelial and monocytic cells. Prior work in neuronal cells provides a rationale that ADAM10 may be involved in the proteolysis of SIRPα. Toth et al. (9) found that neural activity-dependent calcium entry followed by Ca²⁺/calmodulin-dependent protein kinase activation was necessary for SIRPα proteolysis in hippocampal cultures. ADAM10 is canonically activated by calcium influx, plays a role in the degradation of many neuronal substrates including amyloid precursor protein (32), and binds calmodulin, a sensor frequently demonstrated to be activated by the influx of calcium (33). Whether calcium flux modulates SIRPα in myeloid-derived cells requires further investigation.

Although the approximate site of SIRPα proteolysis in the murine immunoreceptor had been traced to the juxtamembrane region in murine SIRPα, this region has very little sequence homology to human SIRPα. Therefore, we performed a series of experiments where we inserted FLAG domain residues in the proximal extracellular membrane that allowed us to determine the approximate region of proteolysis in the human. Surprisingly, human SIRPα cleavage by ADAM10 appeared to be inhibited by the presence of negatively charged residues near

**FIGURE 8. SIRPα proteolysis modulates inflammatory signaling.** a, HeLa cells were incubated with TNFα (10 ng/ml) for the indicated times. Cells were harvested and probed for pIκBα/β and total IκBα. b, HeLa cells were transfected with WT SIRPα and treated with TNFα for 6 h to measure SIRPα proteolysis. c, WT SIRPα- and FLAG-1.5-transfected HeLa cells were treated with TNFα in the presence or absence of Gl. d, HeLa cells were transfected with an empty vector (EV), WT SIRPα, protease-resistant SIRPα (FLAG-1.5), or the proteolysis mutant SIRPα+m or SIRPcyto-C (Cyt-C). At 48 h post-transfection, cells were incubated with TNFα for 15 min. e, HeLa cells were transfected with an empty vector (EV), WT SIRPα, protease-resistant SIRPα mutants (FLAG-1.5 and FLAG-D2), and the enhanced proteolysis mutant (FLAG-R). Cells were treated as described above. f, HeLa cells were transfected with an empty vector (EV), protease-resistant SIRPα (FLAG-1.5), or the proteolysis mutant SIRPα+m (C + M) and treated with TNFα for 4 and 8 h. Cells were then harvested and immunoblotted for pSTAT1 and total STAT1. g, HeLa cells were transfected with empty vector, WT SIRPα, protease-resistant SIRPα (FLAG-1.5), or proteolysis mutant SIRPα+m. At 48 h post-transfection, cells were incubated with TNFα for 6 h, harvested, and treated as above. h, THP-1 cells were preincubated with Gl followed by treatment with LPS for the indicated times. Cells were harvested and probed for pSTAT1 and total STAT1. i, BEAS-2B cells were preincubated with Gl followed by incubation with PA103 (multiplicity of infection, 20) for the indicated times. Cells were harvested and probed for pSTAT1 and total STAT1. Each panel is representative of two to three experiments. Veh, vehicle.
the site of proteolysis, whereas positively charged residues enhanced cutting. This is the first report to our knowledge that suggests a charge-dependent mechanism for MMP-mediated proteolysis. Both human and murine SIRPα have a conserved Gln or Asn near the predicted cleavage site, indicating that amide-containing amino acids in this region might convey ADAM10 specificity. We therefore examined the proteolysis of murine SIRPα overexpressed in HeLa cells. Like hSIRP, mutation of mSIRP Gln to Asp significantly inhibited proteolysis (Fig. 4). However, in both hSIRP and mSIRP, mutation of these residues to Ala neither altered proteolysis nor resulted in decreased inhibition by an ADAM10 inhibitor. This suggests that amide-containing amino acids although centrally located in the cleavage site are unlikely to convey ADAM10 specificity in this system. Additional studies are necessary to determine the structural role of these residues in ADAM-mediated proteolysis.

The molecular interplay between ADAM10 and γ-secretase in other systems led us to hypothesize that SIRPα might also be a target of γ-secretase for sequential processing. This protease belongs to the family of intramembrane-cleaving proteases that target type I membrane proteins. After initial proteolysis, γ-secretase recognizes truncated proteins as a substrate and cleaves the transmembrane domain within the lipid bilayer (17). Several proteins have been described as substrates of γ-secretase cleavage including amyloid precursor protein, Notch, E-cadherin, ErbB4, and CD44 (17). DAPT, a specific γ-secretase inhibitor, prevented disappearance of SIRPα+c in conferring SIRPα as a substrate (35). In addition, we detected a DAPT-sensitive band at the predicted size of a γ-secretase-cleaved SIRPα+c. Secretase cleavage of Notch, amyloid precursor protein, and other proteins leads to nuclear transport of the cytosolic fragment and alteration of gene transcriptional networks (17–19). We hypothesized that a similar mechanism may occur in our system. Interestingly, Shen et al. (25) identified binding partners of SIRPα, found that the COOH-terminal domain specifically interacted with several nuclear transcription factors, lending support to our theory.

In addition to the well studied role of SIRPα in macrophage signaling, we examined whether SIRPα was expressed and proteolytically regulated in the airway epithelium. Yao et al. (27) showed that thrombospondin enhances reactive oxygen species production, leading to renal ischemia-reperfusion injury. SIRPα also protects against cardiac hypertrophy in neonatal rat cardiomyocytes and in murine cardiac tissue (36). Although initial experiments suggested that SIRPα is expressed in the lung, the specific cell types for expression of this immunoreceptor were unclear. Here we observed significant SIRPα expression in a bronchial epithelial cell line (BEAS-2B) and primary human bronchial epithelial cells as well as in an alveolar-like cell line (A549). SIRPα proteolysis was enhanced by bacterial infection in an ADAM10-dependent manner, leading to γ-secretase-mediated secondary proteolysis. This confirms that the myeloid SIRPα and pulmonary SIRPα are similarly regulated and that cleavage of SIRPα may be a widely conserved mechanism in human cells for enhancing inflammatory signaling.

These results beg the question of whether phosphorylation of the SIRPα ITIM domain can alter SIRPα sensitivity to proteolysis. Although a number of stimuli lead to SIRPα phosphorylation, CD47 is the canonical ligand implicated in SIRPα signaling. Ohnishi et al. (8) suggested that ligation of SIRPα by CD47 had no effect on MMP-mediated proteolysis in CHO Ras mutant cells overexpressing SIRPα. However, other SIRPα binding partners may also alter proteolysis. Thrombospondin and surfactant have both been shown to bind SIRPα (27). Surfactant D in particular has been shown to bind the region near the juxtamembrane region and inhibit phagocytosis and therefore is an especially attractive target (37–39).

Ligand binding to SIRPα has been shown to enhance phosphorylation of cytoplasmic ITIM domains through the activation of SRC kinases (4, 40). ITIM phosphorylation recruits phosphatases such as SHP-1 and SHP-2, leading to the inhibition of NF-κB signaling. SIRPα likely interferes with Toll-like receptor signaling specifically by binding SHP-2 and preventing its interaction with IκKs (5). In our experiments, proinflammatory stimuli such as LPS, TNFα, and PA103 resulted in the ADAM10-mediated proteolysis of SIRPα. We also observed that proteolysis-resistant SIRPα inhibited IκKα/β more extensively than did WT SIRPα (Fig. 8). Interestingly, SHP-2 has been shown to be constitutively bound to SIRPα, suggesting that proteolysis may liberate sequestered SHP-2 (5, 7, 41). In addition, studies by Shen et al. (25) identifying binding partners of SIRPα found that the COOH-terminal domain directly bound STAT1, suggesting that SIRPα proteolysis may lead to STAT1 release and nuclear trafficking. Our findings that the SIRPα COOH-terminal domain enhances inflammatory signaling is not without precedent. Neznanov et al. (43), by using an unbiased screen of a retroviral library of randomly fragmented cDNA from mouse fibroblasts, found that a cDNA encoding most of the cytoplasmic domain of SIRPα enhanced NF-κB activation. We have determined that this fragment is produced in vivo in response to inflammatory signals through unique mechanisms (Fig. 9).

Interestingly, inflammatory signaling in neutrophils enhances the truncation of the SIRPα COOH terminus of neutrophils in a serine protease- and IL-17-dependent manner (42). Neutrophils with cleaved SIRPα display an enhanced proinflammatory phenotype in line with our findings, suggesting that SIRPα cleavage is mechanistically relevant and widespread. The authors hypothesized that after serine protease-mediated cleavage of a portion of the COOH-terminal domain the receptor would act as a decoy on the membrane, able to bind SIRPα ligands but unable to signal. Due to the loss of the entire extracellular domain, this mechanism is not at play in our system, but it is plausible, however, that liberation of SHP-2 or STAT1 could be relevant in this model as well through the shedding of COOH-terminally associated SHPs or STAT1. In addition, although serine protease cleavage of SIRPα is observed in neutrophils (42), MMP-mediated cleavage of this receptor in these cells also occurs (13).

In summary, we have demonstrated that SIRPα proteolysis enhances immune activation in response to inflammatory signaling (Fig. 9). Furthermore, we identified a protease that cleaves SIRPα in response to inflammation and the relevant
residues necessary for proteolysis. Finally, γ-secretase cleavage and COOH-terminal fragment release may offer an alternative signaling mechanism for this receptor. Collectively, these data suggest that the proteolytic regulation SIRPα plays an important role in the inflammatory pathway and that specific protease inhibitors may be able to inhibit inflammation.

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