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ER protein 57 (ERp57), a thiol isomerase secreted from vascular cells, is essential for complete thrombus formation in vivo, but other extracellular ERp57 functions remain unexplored. Here, we employed a kinetic substrate-trapping approach to identify extracellular protein substrates of ERp57 in platelet-rich plasma. MS-based identification with immunochromatography confirmation combined with gene ontology enrichment analysis revealed that ERp57 targets, among other substrates, components of the lectin pathway of complement activation: mannose-binding lectin, ficolin-2, ficolin-3, collectin-10, collectin-11, mannose-binding lectin-associated serine protease-1, and mannos-binding lectin-associated serine protease-2. Ficolin-3, the most abundant lectin pathway initiator in humans, circulates as disulfide-linked multimers of a monomer. ERp57 attenuated ficolin-3 ligand recognition and complement activation by cleaving intermolecular disulfide bonds in large ficolin-3 multimers, thereby reducing multimer size and ligand-binding affinity. We used MS to identify the disulfide-bonding pattern in ficolin-3 multimers and the disulfide bonds targeted by ERp57 and found that Cys6 and Cys23 in the N-terminal region of ficolin-3 form the intermolecular disulfide bonds in ficolin-3 multimers that are reduced by ERp57. Our results not only demonstrate that ERp57 can negatively regulate complement activation, but also identify a control mechanism for lectin pathway initiation in the vasculature. We conclude that extensive multimerization in large ficolin-3 multimers leads to a high affinity for ligands and strong complement-activating potential and that ERp57 suppresses complement activation by cleaving disulfide bonds in ficolin-3 and reducing its multimer size.

Among other functions, thiol isomerases catalyze the folding of nascent proteins in the endoplasmic reticulum through the oxidation, reduction, or isomerization of disulfide bonds (1). Protein-disulfide isomerase (PDI)3 is the prototypic enzyme in this protein family that includes about 20 members. They are characterized by conserved thioredoxin-like structural folds harboring a Cys-Gly-His-Cys (CGHC) motif (2). A subset of known thiol isomerases are actively secreted from vascular cells upon their activation (3, 4), and a role for some of these thiol isomerases in thrombus formation has been established. PDI is released from platelets and endothelial cells and is required for platelet thrombus formation and fibrin deposition in vivo (5, 6).

ERp57 is a close homolog of PDI and is important for platelet function and required for normal thrombus formation in vivo (7–9). In addition, similar roles for ERp5 (10) and ERp72 (11, 12) are known. This group of enzymes are considered the vascular thiol isomerases (13).

The mechanism by which thiol isomerases control thrombus formation or their roles in other processes in the vasculature are not well understood. To address these questions, we have adapted a kinetic substrate trapping strategy to identify substrates of extracellular thiol isomerases. Substitution of the C-terminal cysteine in the CGHC active sites to alanine results in a trapping variant that forms a stable covalent disulfide link with its substrate (14). Using this methodology, we identified plasma vitronectin as one of the substrates of PDI (15). Cleavage of one or two disulfide bonds in vitronectin by PDI enables binding of vitronectin to β3 integrins on the endothelium and support of thrombus formation.

In the current study, we extended this approach using the kinetic substrate trapping strategy to identify extracellular targets of ERp57. Like PDI, ERp57 has four domains in the a-b-a′-b′ configuration, where the a and a′ domains have an active-site CGHC motif (16, 17). ERp57 and PDI have 33% sequence similarity. Among the ERp57 substrates identified were mannos-binding lectin (MBL), ficolin-2, ficolin-3, collectin-10, collectin-11, mannose-binding lectin-associated serine protease-1 (MASP-1), and mannose-binding lectin-associated serine protease-2 (VBS, veronal-buffered saline; 12C-IPA, 2-iodo-N-phenylacetamide; MPB, biotin-conjugated maleimide; AMC, 7-amino-4-methylcoumarin.

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3 The abbreviations used are: PDI, protein-disulfide isomerase; MBL, mannos-binding lectin, MASP, mannos-binding lectin-associated serine protease; VBS, veronal-buffered saline; 12C-IPA, 2-iodo-N-phenylacetamide; MPB, biotin-conjugated maleimide; AMC, 7-amino-4-methylcoumarin.
protease-2 (MASP-2). These proteins are among the components of the lectin pathway of complement activation, one of the three pathways that initiate the complement system. Ficolin-3, a multimeric pattern recognition molecule that is the most abundant lectin pathway initiator in human plasma, is susceptible to disulfide bond cleavage by ERp57. ERp57 reduces large ficolin-3 multimers and attenuates lectin pathway initiation by inhibiting target recognition by ficolin-3. Our findings reveal that ERp57 targets complement activation via the lectin pathway.

Results

The lectin pathway of complement activation is targeted by ERp57

To identify vascular substrates of ERp57, we adapted the kinetic substrate trapping strategy described for PDI (15). We generated and purified recombinant ERp57 variants where both of the CGHC active sites were mutated to CGHA, termed ERp57-CACA (Fig. 1A). An inactive variant with both active sites as AGHA, termed ERp57-AAAA, served as a negative control. These ERp57 variants were evaluated in the insulin reduction assay (Fig. 1B, left) and the di-eosin-GSSG assay (Fig. 1B, right). ERp57-AAAA had no enzymatic activity, and the activity of ERp57-CACA was reduced. When added to activated platelet-rich plasma, the ERp57-CACA variant formed disulfide-linked complexes with a number of substrates, as evidenced by high-molecular weight complexes on unreduced gels (Fig. 1C, left) that were resolved by reduction with DTT. The eluates were precipitated with TCA and analyzed by MS. The graph shows gene ontology biological process enrichment analysis of MS-identified proteins in the ERp57-CACA data set, using the plasma proteome as background. Enriched ontologies with a false discovery rate < 0.05 are shown, with selected ontologies indicated.
ERp57 regulates the lectin pathway

Focused on the 76 proteins exclusively detected in the ERp57-CACA sample. We subjected the ERp57-CACA data set to gene ontology enrichment analysis (18, 19) using the PANTHER database (20) and gene ontology biological process annotations, with the plasma proteome (21) as background. The analysis revealed an enrichment of the lectin pathway of complement (GO:0001867, -fold enrichment 30, false discovery rate 0.0014) (Fig. 1D). A manual search for components of the lectin pathway (22) in the MS data set revealed that all components of this pathway were detected with the ERp57-CACA trapping variant except for ficolin-1 (Table 1), demonstrating that this pathway is targeted by ERp57.

The lectin pathway is initiated by six different pattern recognition molecules: MBL, ficolin-1, -2, and -3, and collectin-10 and -11, that recognize carbohydrate moieties on microorganisms and damage-associated molecular patterns on altered-self structures (22). Of these, ficolin-3 is the most abundant and potent lectin pathway initiator in humans (23). Substrate trapping of MBL, ficolin-2, and ficolin-3 by ERp57-CACA was confirmed by immunoblotting. These proteins circulate in plasma as disulfide-linked multimers and display a ladder of bands when analyzed on unreduced SDS gels (Fig. 2, A–D, top). In contrast, under reducing conditions, only the monomers of MBL, ficolin-2, and ficolin-3 were observed (Fig. 2, A–D, bottom). ERp57-CACA selectively trapped large multimers of the ficolins and MBL. When comparing densitometry lane profiles on unreduced SDS gels, a shift in apparent molecular weight was seen only for the largest species, indicating that they form disulfide-linked complexes with the ERp57 trapping variant (Fig. 2, E–G). These results confirm the substrate identification from MS and show that ERp57 targets the multimeric forms of lectin pathway recognition molecules circulating in plasma.

**ERp57 attenuates ficolin-3–dependent lectin pathway initiation**

To determine whether ERp57 has functions that regulate complement activation through the lectin pathway, we used an ELISA with lectin pathway activators coated on the solid phase and serum as a source of complement proteins. ERp57-CCCC was added to the reactions in the presence of 10 μM DTT, and ligand recognition by MBL, ficolin-2, and ficolin-3 was evaluated. MBL binding was monitored on a matrix consisting of the yeast polysaccharide mannnan (24) and was not altered by ERp57 (data not shown). Compared with MBL, ficolins have a different ligand specificity and bind strongly to acetylated compounds, such as acetylated BSA (25, 26). A time- and concentration-dependent inhibition of ficolin-3 by ERp57 was observed, and binding to the acetylated BSA matrix was reduced to ~30% of control (Fig. 3A). Reduction in ficolin-3 binding required the CGHC active-site motifs; ERp57-AAAA had no effect (Fig. 3B). In addition, ERp57-CCCC had a moderate but significant inhibitory effect on ficolin-2 binding to acetylated BSA (data not shown). These results demonstrated that ERp57 inhibits ligand recognition by the ficolin-3–dependent lectin pathway.

Ficolin-3 circulates in complex with serum proteases (MASP-1 and MASP-2) that are activated through a conformational change following target binding. We monitored MASP-1 activity by cleavage of the synthetic substrate VPR-AMC (27) when ficolin-3 was captured on acetylated BSA. ERp57-CCCC decreased VPR-AMC cleavage in a dose-dependent fashion (Fig. 4A). No inhibition was seen with the inactive ERp57-AAAA (Fig. 4B). A bar graph summarizes these results (Fig. 4D).

Initiation of the lectin pathway leads to activation of complement factors C2 and C4 by MASP-1 and MASP-2. Activated C2 and C4 in turn form a C3 convertase (C2aC4b) that activates complement factor C3 and leads to propagation of complement activation. The ELISA was used to measure downstream complement activation on acetylated BSA, which is specific for ficolin-3 with only a minor contribution from ficolin-2 (26). The effect of ERp57-CCCC on ficolin-3 binding and ficolin-3–MASP activity was paralleled by a decrease in complement activation with a reduction in C4 deposition observed in the presence of ERp57 (Fig. 5A, left). Similarly, decreased C3 deposition on acetylated BSA was observed in the presence of ERp57 (Fig. 5B, right). Taken together, ERp57 attenuates ficolin-3 target recognition, the proteolytic activity of the ficolin-3–MASP complex, and downstream complement activation.

**ERp57 cleaves ficolin-3 multimers by disulfide reduction**

Circulating ficolin-3 forms a range of multimers, with the largest species having biological activity (23). To determine whether the suppressive effect of ERp57 on lectin pathway initiation was due to direct changes in ficolin-3 multimerization, we performed SDS-PAGE and immunoblotting under nonre-
ERp57 regulates the lectin pathway

When serum was incubated with ERp57-CCCC, large ficolin-3 multimers disappeared with a concomitant increase in intermediately sized multimeric species (Fig. 6, A and B). ERp57-AAAA had no effect on the multimer pattern. The shift in multimerization indicated that ficolin-3 was cleaved by ERp57. ERp57 also cleaved ficolin-2 multimers, whereas minimal MBL cleavage was seen (data not shown).

Ficolin-3 purified from serum showed multimeric forms up to 21-mer on unreduced gels. When incubated with ERp57-CCCC, we observed a shift in multimer size in purified ficolin-3 (Fig. 6C, right). As ficolin-3 forms multimers through disulfide bonds, we hypothesized that ficolin-3 cleavage by ERp57 occurred through disulfide bond reduction and used biotin-conjugated maleimide (MPB) to label free thiols (Fig. 6C, left).

ERp57 increased MPB incorporation into ficolin-3, an effect that was correlated to the decrease in multimer size. On unreduced gels, MPB labeling of ficolin-3 species of intermediate sizes was observed, indicating that these were formed after reductive cleavage of large multimers.

**ERp57 cleaves ficolin-3 by reduction of intersubunit disulfide bonds formed by the cysteines Cys^6 and Cys^23**

The ficolin-3 monomer contains 8 cysteine residues. Two cysteines (Cys^6 and Cys^23) near the N terminus have been predicted to mediate multimerization through intermolecular disulfide bonds with other subunits (29), but the specific multimerization pattern in native ficolin-3 is not known. We used MS to detect disulfide-bridged tryptic peptides in order to map the specific intermolecular disulfide bonds in ficolin-3. Pep-

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**Figure 2. Immunoblotting against identified putative substrates.** Trapping experiments were performed in platelet-rich plasma with ERp57-CACA or ERp57-AAAA (50 μg). Samples were analyzed by gel electrophoresis and Western blotting under nonreducing (top) or reducing conditions (bottom) using goat anti-MBL (A), goat anti-ficolin-2 (B), goat anti-ficolin-3 (C), and mouse anti-FLAG (D) antibodies. E–G, the unreduced blots shown in A–C were analyzed by densitometry, and the lane profiles of platelet-rich plasma (green) and ERp57-CACA (blue) were overlaid. E, MBL; F, ficolin-2; G, ficolin-3. Arrows indicate multimers with a shift in molecular weight indicative of complex formation with ERp57-CACA. n = 3; representative experiments are shown.

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ERp57 regulates the lectin pathway

Figure 3. ERp57 inhibits ficolin-3 ligand recognition. A, the binding of ficolin-3 to acetylated BSA was detected using an ELISA. Diluted serum was preincubated for 5 min with ERp57 in the presence of 10 μM DTT and added to plates coated with 0.5 μg/ml acetylated BSA for the times indicated. Green, serum alone; red, DTT; blue, 0.1 μM ERp57-CCCCC; brown, 0.5 μM ERp57-CCCC. B, ficolin-3 binding to acetylated BSA-coated plates was performed as above. ERp57 and PDI variants were added as indicated, and plates were incubated with samples for 30 min at 37 °C. Binding of ficolin-3 is shown for ERp57-CCCC, ERp57-AAAA, and PDI-CCCC. n = 3–5; data are presented as mean ± S.E. (error bars). *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001 (one-way ANOVA with Dunnett's post-test correction).

Figure 4. ERp57 attenuates the serine protease activity of the ficolin-3–MASP complex. Ficolin-3–MASP complexes were captured for 30 min at 37 °C from 1% serum on plates coated with 5 μg/ml acetylated BSA. The proteolytic activity of the captured complexes was evaluated by cleavage of the fluorogenic substrate VPR-AMC. A, ERp57-CCCCC. Green, serum alone; red, DTT; blue, 0.1 μM ERp57-CCCCC; brown, 0.5 μM ERp57-CCCCC; black, serum in the absence of acetylated BSA matrix. B, ERp57-AAAAA. Green, serum alone; red, DTT; yellow, 0.1 μM ERp57-AAAA; orange, 0.5 μM ERp57-AAAAA; black, serum in the absence of acetylated BSA matrix. C, PDI-CCCCC. Green, serum alone; red, DTT; light purple, 0.1 μM PDI-CCCCC; dark purple, 0.5 μM PDI-CCCCC; black, serum in the absence of acetylated BSA matrix. D, bar graph showing the mean ± S.E. (error bars) of four experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001 (one-way ANOVA with Dunnett's post-test correction). RFU, relative fluorescence units.

tides bridged by disulfides connecting Cys6 to Cys6′ and Cys23 to Cys23′ were detected in ficolin-3 dimers, trimers, and higher oligomeric forms but not in the ficolin-3 monomer (Fig. 7). Monomeric ficolin-3 contained an intramolecular disulfide bond between Cys6 and Cys23. Cys6 and Cys23 were also linked in a disulfide bond in multimeric ficolin-3, which has to be intermolecular, as ficolin-3 with an intramolecular Cys6–Cys23 disulfide cannot be linked to other ficolin-3 subunits. No other intermolecular disulfide bonds were found. We conclude that ficolin-3 multimers are assembled through a mixture of Cys6–Cys6′, Cys23–Cys23′, and Cys6–Cys23′ intermolecular disulfide bonds. ERp57 cleaves ficolin-3 multimers by reduction of these disulfide bonds.

Estimation of the redox potential of ficolin-3 multimers

To assess the properties of the ficolin-3 intermolecular disulfide bonds involved in multimerization, purified serum ficolin-3 was equilibrated in GSH redox buffers with varying ratios of reduced (GSH) to oxidized (GSSG) glutathione (GSH2/GSSG ratio). We used multimer distribution as an indirect measure of formation of the Cys6–Cys6′, Cys23–Cys23′, and Cys6–Cys23′ intermolecular disulfide bonds and quantified the
abundance of each multimer compared with ficolin-3 without GSH. A shift in ficolin-3 multimerization and a gradual disappearance of large multimers occurred at increasing ratios of reduced to oxidized GSH, demonstrating reduction of the intermolecular disulfide bonds (Fig. 8, A and B). When the fraction of reduced to oxidized multimers was plotted against the GSH²/GSSG ratio, the redox potentials could be calculated from the obtained equilibrium constants. These were found to be −175 mV for the 21-mer, −188 mV for the 18-mer, −194 mV for the 15-mer, and −207 mV for the 12-mer (Fig. 8C). This demonstrates that the redox potentials of ficolin-3 multimers are a function of multimer size, with the largest ficolin-3 species having the highest redox potential.

**Discussion**

Studies in mouse models have revealed an important role for extracellular ERp57 in thrombus formation (7–9). Additional functions of extracellular ERp57 have been proposed. A role for thiol–disulfide exchange mediated by ERp57 in sperm–gamete fusion has been reported (30), and ERp57 was recently shown to modulate the activity of transglutaminase 2 secreted by endothelial cells (31).

In the current study, we present evidence that extracellular ERp57 can also regulate complement initiation. Based upon a substrate trapping strategy and gene ontology enrichment analysis, we identified the lectin pathway of complement activation as a pathway regulated by ERp57. We demonstrate that ERp57 targets plasma ficolin-3, the most abundant lectin pathway initiator in humans (23), and attenuates ficolin-3–dependent complement activation by cleaving large ficolin-3 multimers.

Our results thus show that in addition to modulating thrombus formation, an additional function of ERp57 in the vasculature may be to regulate complement initiation. In agreement with these findings, a suppressive effect of the protein reductant thioredoxin on the late stages of complement activation was demonstrated, suggesting a general role of redox reactions in complement regulation. Thioredoxin did not affect the lectin pathway, indicating a nonoverlapping role with ERp57 in complement regulation (32).

Complement activation initiates a potent host defense pathway that leads to destruction and removal of its presumably foreign target. Damaging mutations in complement regulators in humans are associated with conditions characterized by tissue destruction mediated by autologous complement attack, such as atypical hemolytic uremic syndrome (33). These observations indicate that it is critical for host cells to protect themselves from complement recognition. Based on our results, we propose that ERp57 protects the vasculature from complement-mediated damage and serves to limit recognition by the lectin pathway. However, ligands for ficolin-3 are not well-characterized. Ficolin-3 can recognize late apoptotic cells (34), but its potential to interact with host cells in the vasculature remains to be determined. Nevertheless, in accord with this hypothesis, platelets and endothelial cells are known to express high levels of complement regulators on their surface (35) (36). Regulation of complement activation by von Willebrand factor demonstrates that a factor that is prothrombotic can also serve to limit complement attack on host cells (37, 38).

The kinetics of ERp57 action on ficolin-3 are slow as measured in our in vitro studies. The relative concentrations of the enzyme and substrate, whether ERp57 acts on other critical substrates in the lectin pathway or whether ERp57 requires chaperones for enhancing the rate of inactivation, need to be explored in more physiologic systems—perhaps in future studies using an animal model. A challenge for such studies will be the significant differences in the lectin pathway between mice and humans and the fact that the gene encoding ficolin-3 is inactivated to a pseudogene in the rodent lineage (39). Instead, MBL is the dominating lectin pathway trigger in mice, with the main mouse MBL variant MBL-C having up to 100-fold higher plasma concentration than MBL in humans (40). In this context, it should be noted that ERp57 did not affect MBL function in our studies.

The biological activity of lectin pathway pattern recognition molecules is a function of their multimer size. Multimeric species show a high affinity for ligands due to their multiple lectin domains compared with a single lower-affinity binding site in the monomeric subunits (41). MASP3s preferentially interact with large multimers (42). These conclusions are mostly based on studies on MBL but are likely to apply to ficolins as well, given their structural similarity. Ficolin-3 forms up to 21-mers of the basic subunit, compared with MBL (42) and ficolin-2 (23), which assemble into 9-mers and 12-mers, in accordance with observations that ficolin-3 is the most potent lectin pathway activator (23). Our results suggest a novel mechanism to inhibit the initiation of the lectin pathway through reductive cleavage of multimeric ficolin-3 by ERp57 into smaller inactive forms. Multimer cleavage as a means to control protein activity

**Figure 5. ERp57 attenuates ficolin-3-dependent complement activation.** Diluted sera and ERp57 including DTT were added to plates coated with 0.5 μg/ml acetylated BSA for the indicated time points at 37 °C. Complement activation was assessed by developing plates for deposition of complement components C4 (A) or C3 (B). Green, serum alone; red, DTT; blue, 0.1 μM ERp57-CCCC; brown, 0.5 μM ERp57-CCCC. n = 3; data are presented as mean ± S.E. (error bars), * p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001 (one-way ANOVA with Dunnett’s post-test correction).
is exemplified by proteolytic cleavage of von Willebrand factor by ADAMTS13, which is essential to prevent intravascular thrombosis (43). Disulfide reduction has been proposed as an additional mechanism to control von Willebrand factor multimer size (44, 45). Hence, cleavage of disulfide bonds may be a general mechanism to regulate multimeric proteins.

Our MS analyses revealed that the two N-terminal cysteines Cys-6 and Cys-23 form the intermolecular disulfide bonds that mediate multimerization in native ficolin-3. Estimation of their redox potential showed a multimerization-dependent effect, indicative of ERp57 favoring the large biologically active ficolin-3 species. A feature of intermolecular disulfide bonds is the tensile strain that is imposed on these bonds. Force-induced strain can destabilize the oxidized state to facilitate disulfide bond cleavage (46, 47). Mechanical strain in large ficolin-3 multimers could be an explanation for the susceptibility of these intermolecular disulfide bonds to reduction and the reason why ERp57 preferentially cleaves highly multimeric ficolin-3.

In conclusion, our results demonstrate a novel role for ERp57 as a regulator of the lectin pathway of complement activation. Cleavage of intermolecular disulfide bonds in the multimeric pattern recognition molecule ficolin-3 by ERp57 prevents ligand recognition and complement initiation through this pathway. This study reveals novel functions of a vascular thiol isomerase and adds to the list of proteins regulated through allosteric disulfide bonds (48).

Figure 6. ERp57 alters ficolin-3 multimer distribution by disulfide reduction. A, serum was incubated with ERp57 variants and 10 μM DTT for 30 min at 37 °C. Samples were analyzed for ficolin-3 multimerization after separation in unreduced SDS gels and immunoblotting with goat anti-human ficolin-3 antibodies (top). ERp57 variants were detected via their FLAG epitope to demonstrate that equal amounts were present in the reactions at the respective concentrations (bottom). B, densitometry analysis of the ficolin-3 immunoblot shown in A. Green, serum alone; red, DTT; light blue, 0.1 μM ERp57-CCCC; dark blue, 0.5 μM ERp57-CCCC; yellow, 0.1 μM ERp57-AAAA; orange, 0.5 μM ERp57-AAAA. C, purified ficolin-3 (25 μg/ml) was incubated with 0.5 μM ERp57 (30 μM/m) in the absence or presence of 5 μM DTT for 15 min and then labeled with MPB. Samples were analyzed by gel electrophoresis and Western blotting. MPB incorporation was detected using horseradish peroxidase–labeled streptavidin (left) and ficolin-3 with goat anti-human ficolin-3 antibodies (right). Asterisks indicate ERp57 variants that are also labeled by MPB. Unreduced (top) and reduced gels (bottom) are shown. D, densitometry analysis of the ficolin-3 immunoblot shown in C. Green, serum alone; red, 0.5 μM ERp57-CCCC; blue, DTT; brown, DTT and 0.5 μM ERp57-CCCC. n = 3; representative experiments are shown.
Experimental procedures

Materials

Goat anti-human ficolin-3 (AF2367, R&D Systems), biotinylated goat anti-human ficolin-3 (BAF2367, R&D Systems), goat anti-human MBL (AF2307, R&D Systems), biotinylated goat anti-human MBL (BAF2307, R&D Systems), goat anti-human ficolin-2 (AF2428, R&D Systems), biotinylated goat anti-human ficolin-2 (BAF2428, R&D Systems), biotinylated rabbit anti-human C4b (11254-05021, AssayPro), goat anti-human C3 (A213, Complement Technology), and mouse anti-FLAG (8146, Cell Signaling Technology) were purchased from commercial sources. Platelet-rich plasma and serum were obtained from healthy donors. The serum was pooled, aliquoted, and stored at −80 °C until use. All blood donors provided informed consent, and the study was approved by the Beth Israel Deaconess Medical Center Committee on Clinical

Figure 7. Ficolin-3 multimerization is mediated by disulfide bonds between the N-terminal cysteines Cys⁶ and Cys²³. N-terminal domain disulfide-linked peptides involving Cys⁶ and Cys²³ were quantified in purified ficolin-3 subjected to a single alkylation step with [¹²C]IPA and separated by gel electrophoresis in unreduced gels. Ficolin-3 monomers, dimers, trimers, tetramers, and higher-order multimers were excised from the gel and analyzed by MS. Peptide abundance is expressed relative to the invariant peptide linked by Cys⁶³ and Cys⁸⁷ in the collagen-like domain of ficolin-3. Black, monomer; green, dimer; red, trimmer; blue, tetramer; brown, multimeric. A, N-terminal ficolin-3 disulfide-linked peptides involving Cys⁶ and Cys²³. B, aberrant ficolin-3 disulfide-linked peptides involving Cys⁶ and Cys²³. Note the difference in the magnitude of the y axis of about 100-fold.

Figure 8. Estimation of the redox potential of ficolin-3 multimers. A, purified ficolin-3 was equilibrated in nitrogen-flushed GSH buffers with varying ratios of reduced to oxidized glutathione (GSH²/GSSG ratio) for 16 h at 25 °C, pH 7. Reactions were stopped by alkylation with iodoacetamide, and ficolin-3 multimerization as a measure of the redox state of the intermolecular disulfide bonds was analyzed by unreduced SDS-PAGE and Western blotting. GSH²/GSSG ratios are indicated above the gel image, and multimer size is shown to the left. n = 2; a representative blot is shown. B, the abundance of ficolin-3 21-mers, 18-mers, 15-mers, and 12-mers was quantified by densitometry at different GSH²/GSSG ratios and expressed as percentage of ficolin-3 abundance in the absence of GSH. Error bars, S.E. (error bars). Shown are ficolin-3 21-mer (green), 18-mer (red), 15-mer (blue), 12-mer (brown). C, the fractions of reduced ficolin 21-mer, 18-mer, 15-mer, and 12-mer were plotted against the GSH²/GSSG ratio and fitted to Equation 1. Redox potentials were determined from Equation 2 using the calculated equilibrium constants. Shown are ficolin-3 21-mer (green), 18-mer (red), 15-mer (blue), and 12-mer (brown).
ERp57 regulates the lectin pathway

Investigations and is in compliance with the Helsinki principles. Other chemicals and reagents were from Sigma-Aldrich unless noted otherwise.

Kinetic mechanism–based substrate trapping

These experiments were performed according to Bowley et al. (15) with modifications. The cDNA of human ERp57 was cloned into the pT7-FLAG-SBP expression vector and used to generated three classes of recombinant ERp57 constructs using site-directed mutagenesis: 1) WT ERp57, containing 57CGH60 and 406CGH409 active sites located in the a domain and a’ domain, respectively (ERp57−CCCC); 2) the trapping variant in which an active-site cysteine is mutated to alanine, precluding cleavage of the ERp57−substrate complex, 57CGH60 and 406CGH409 (ERp57−CACA); and 3) inactive ERp57 in which cysteines were replaced by alanines, 57AGH60 and 406AGH409 (ERp57−AAAA). The proteins were expressed in BL21 Escherichia coli bacteria and purified by two cycles of affinity chromatography, using streptavidin-coated agarose beads (Thermo Fisher) and then agarose beads conjugated to a monoclonal FLAG antibody. The purity of the recombinant proteins was assessed by Coomassie staining, and the catalytic function was assessed by insulin reduction (49) and di-eosin-GSSG (50) assays.

For the kinetic trapping experiments, platelet-rich plasma anticoagulated with 4% citrate was obtained from blood donors. ERp57 variants (50 μg) were reduced with DTT and excess DTT removed with Zeba desalting columns (Thermo Fisher). Prereduced ERp57 variants were added to 1 ml of plateletrich plasma substituted with a 5 mM concentration of the fibrin polymerization inhibitor Gly-Pro-Arg-Pro, and the reactions were activated with 0.4 units/ml thrombin. After proceeding for 3 min, reactions were stopped with hirudin and N-ethylmaleimide, and ERp57 complexed to substrates was precipitated using streptavidin-coated agarose beads. Proteins were eluted from the beads by boiling in sample buffer and then analyzed by SDS-PAGE and Western blot analysis under either nonreducing or reducing conditions. For MS analysis, a trapping experiment was performed with ERp57−CACA and ERp57−AAAA. Disulfide-linked substrates were eluted with 10 mM DTT, and the eluate was precipitated with TCA and then subjected to LC/MS/MS analysis on an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific) after trypsin digestion. Peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences were determined by matching protein databases with the acquired fragmentation pattern with Sequest (Thermo Fisher Scientific).

Solid-phase lectin pathway assays

For functional assessment of the lectin pathway, a solid-phase ELISA format was used. Maxisorp 96-well plates were coated overnight at 4 °C with 0.5 μg/ml acetylated BSA as a ligand for ficolin-2 (25) and ficolin-3 (26) and with 10 μg/ml mannan as a ligand for MBL (24). Plates were then washed with TBS, 0.05% Tween 20 with 5 mM CaCl2 (TBS-T-Ca) and blocked with Veronal-buffered saline (Boston Bioproducts) supplemented with 2 mM CaCl2, 2 mM MgCl2, and 0.05% Tween 20 (VBS-T). After blocking and washing of plates, serum was diluted 1:100 in VBS-T and mixed with ERp57 variants reduced with 10 μM DTT. Samples were preincubated for 5 min at 37 °C before they were added to the plates and incubated at 37 °C for the indicated times. Binding of ficolin-2, ficolin-3, or MBL was detected using biotinylated antibodies and horseradish peroxidase−conjugated N-avidin (Thermo Fisher). The plates were developed using True Blue Peroxidase substrate (SeraCare), terminated with 1 M HCl, and absorbances were read at 450 nm using a Spectramax plate reader. No binding of ficolin-2 and ficolin-3 was observed to unmodified BSA or to mannan, and MBL bound specifically to mannan but not to acetylated or unmodified BSA.

Ficolin-3–mediated complement activation was assessed using an ELISA with acetylated BSA-coated plates by measuring deposition of complement components C4 and C3. Serum diluted 1:100 was mixed with ERp57 variants and DTT before being added to the plates. To minimize interference from the alternative and classical pathways of complement activation, serum was preincubated for 5 min on ice with 0.1 mg/ml sodium polyethanol sulfate, an inhibitor of the alternative pathway and classical pathway at these concentrations. The lectin pathway was preserved (51). Sodium polyethanol sulfate did not affect ficolin-3 binding, and no C4 or C3 deposition occurred on unmodified BSA in the presence of sodium polyethanol sulfate.

The proteolytic activity of the ficolin-3−MASP complexes was assessed by cleavage of the fluorogenic substrate N-tert-butoxycarbonyl-VPR-AMC (R&D Systems), which is cleaved by MASP-1 (27). Ficolin-3−MASP-1 complexes were captured on acetylated BSA-coated plates (5 μg/ml) from serum diluted in VBS-T with or without ERp57 variants and DTT for 30 min at 37 °C. Plates were washed, and VPR-AMC diluted to 200 μM in VBS-T was added. Substrate cleavage was monitored on a Biotek fluorescen plate reader with excitation at 380 nm and emission at 460 nm.

Purification of ficolin-3

Ficolin-3 was purified from serum obtained from the blood of donors (28). CaCl2 (10 mM) was added to citrated plasma and clotted with 10 mM, and the supernatant was precipitated using PEG with a 4–8% cut-off. The pellet was dissolved in TBS-T-Ca, and ficolin-3 was affinity-purified on a column of acetylated human serum albumin conjugated to agarose. Protein was eluted with 1 M sodium acetate and dialyzed against TBS-Ca. Co-purified IgG and IgM were removed using protein A- and anti-IgM-agarose columns, and the eluate was concentrated using Vivaspin 6 spin columns (GE Healthcare) and then aliquoted and stored at −80 °C.

MS analysis of ficolin-3

For analysis of disulfide pairing by MS, purified ficolin-3 (10 μg) was alkylated with 5 mM 2-iodo-N-phenylacetamide ([12C]-IPA) in the dark for 1 h at 25 °C and resolved on SDS-PAGE in the absence of reducing agent. The gel slices were washed and dried before digestion of proteins with 12 ng/μl chymotrypsin (Roche Applied Science) in 25 mM NH4CO2 and 10 mM CaCl2 for 4 h at 37 °C followed by 12 ng/μl trypsin (Promega) overnight at 25 °C. Reactions were stopped by adding 5% (v/v) for-
mic acid, and peptides were eluted from the gel slices with 5% formic acid and 50% (v/v) acetonitrile.

Using a Thermo Fisher Scientific Ultimate 3000 HPLC, peptides in 0.1% formic acid (final volume 12 μl) were resolved on a 35 cm × 75 μm C18 reverse phase analytical column using a 2–35% acetonitrile gradient over 22 min with a flow rate of 300 nl/min. The peptides were ionized by electrospray ionization at +2.0 kV. Tandem MS analysis was carried out on a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific) using CID fragmentation. The data were searched using Mascot (Matrix Science) against the Uniprot database. Ion abundance of peptides was calculated as described (52). Briefly, extracted ion chromatograms were generated using XCalibur Qual Browser, and area was calculated using the automated peak detection function. The abundance of disulphide-linked peptides was expressed as a ratio relative to an invariant disulphide-linked peptide between Cys^{63} and Cys^{87}.

Estimation of the redox potential of ficolin-3 multimers

Purified ficolin-3 (50 μg/ml) was incubated with nitrogen-flushed PBS, pH 7, with 1 mM EDTA and varying concentrations of reduced (GSH) and oxidized (GSSG) glutathione for 16 h at 25 °C. Reactions were terminated by alkylation with 25 mM iodoacetamide, and ficolin-3 multimerization was analyzed by SDS-PAGE in the absence of reducing agent and Western blotting. The results were expressed as the ratio of reduced to oxidized protein and fitted to Equation 1,

\[
R = \frac{[\text{GSH}]^2}{[\text{GSSG}]} \quad \left(\text{Eq. 1}\right)
\]

where \( R \) is the fraction of reduced protein at equilibrium and \( K_{eq} \) is the equilibrium constant. The standard redox potentials (\( E^{\circ} \)) of the intermolecular disulphide bonds in ficolin-3 multimers were calculated using the Nernst equation (Equation 2),

\[
E^{\circ} = E^{\circ}_{\text{GSSG}} - \frac{RT}{2F} \ln K_{eq} \quad \left(\text{Eq. 2}\right)
\]

using a value of −240 mV for the standard redox potential of the GSSG disulphide bond.

Statistics

Results are presented as mean ± S.E. To test for difference between experimental conditions, one-way ANOVA with Dunnett’s post-test correction was used. \( p < 0.05 \) was considered statistically significant. \( p \) values are indicated as follows: *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \); ****, \( p < 0.0001 \).

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