PRELP Protein Inhibits the Formation of the Complement Membrane Attack Complex[*]

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Background: PRELP binds the complement inhibitor C4b-binding protein and may have other complement regulatory functions.
Results: PRELP inhibits the formation of the membrane attack complex and thereby inhibits all three pathways of complement.
Conclusion: PRELP regulates complement responses at several levels of the cascade.
Significance: PRELP may act as a local complement inhibitor at basement membranes or at sites with exposed cartilage.

PRELP is a 58-kDa proteoglycan found in a variety of extracellular matrices, including cartilage and at several basement membranes. In rheumatoid arthritis (RA), the cartilage tissue is destroyed and fragmented molecules, including PRELP, are released into the synovial fluid where they may interact with components of the complement system. In a previous study, PRELP was found to interact with the complement inhibitor C4b-binding protein, which was suggested to locally down-regulate complement activation in joints during RA. Here we show that PRELP directly inhibits all pathways of complement by binding C9 and thereby prevents the formation of the membrane attack complex (MAC). PRELP does not interfere with the interaction between C9 and already formed C5b-8, but inhibits C9 polymerization thereby preventing formation of the lytic pore. The alternative pathway is moreover inhibited already at the level of C3-convertase formation due to an interaction between PRELP and C3. This suggests that PRELP may down-regulate complement attack at basement membranes and on damaged cartilage and therefore limit pathological complement activation in inflammatory disease such as RA. The net outcome of PRELP-mediated complement inhibition will highly depend on the local concentration of other complement modulating molecules as well as on the local concentration of available complement proteins.

The complement system is an essential part of our innate immunity and acts as a defense against pathogens as well as an endogenous danger sensor. Unbalanced or misdirected complement activation can have significant roles in both acute and chronic inflammatory conditions such as rheumatoid arthritis (RA),[2] multiple sclerosis or systemic lupus erythematosus. Several reports demonstrate that proteins in the extracellular matrix, among others members of the mainly cartilage-resident small leucine-rich repeat protein (SLRP) family, can trigger complement activation, thereby disturbing the balance in the triggering and control of complement leading to pathological activation. This has been proposed to contribute to the disease progression in RA where these molecules that normally are sequestered within the cartilage suddenly become exposed to complement due to tissue degradation. The SLRPs fibromodulin, osteoadherin, and chondroadherin all bind and activate the classical complement pathway initiator C1q, whereas decorin and biglycan bind C1q while inactivating the molecule (1–3). These molecules further regulate the level of their initiated complement response by binding soluble complement inhibitors such as C4b-binding protein (C4BP) and factor H (3, 4).

The complement system can be activated through three distinct pathways that merge at the level of the C3-convertase (Fig. 8). The classical pathway is triggered by immune complexes and several endogenous ligands including C-reactive protein (CRP) (5), whereas the lectin pathway is mainly activated by certain sugar residues on pathogenic surfaces. The alternative pathway is triggered by properdin or by spontaneous hydrolysis of C3, rendering a molecule capable of participating in the formation of the alternative C3-convertase. Cleavage of C3 by these convertases leads to the formation of C3b, which by associating with the C3-convertase changes its enzymatic specificity to C5. Cleavage of C5 results in release of C5a, which promotes anaphylaxis, whereas C5b forms the base for the assembly of the lytic membrane attack complex (MAC), composed of C5b, C6, C7, C8, and several molecules of C9 (for review, see Ref. 6). The main inhibitory regulation of the complement pathways occurs at the C3- and C5-convertases,

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2 The abbreviations used are: RA, rheumatoid arthritis; A1AT, α-1-antitrypsin; CRP, C-reactive protein; C4BP, C4b-binding protein; MAC, membrane attack complex; NHS, normal human serum; PRELP, proline arginine-rich end leucine-rich repeat protein; SLRP, small leucine-rich repeat protein; TCA, trichloroacetic acid.

whereas formation of the MAC is inhibited by CD59, vitronectin (S-protein), and clusterin (apolipoprotein J or SP-40) (7–9).

PRELP (proline/arginine-rich end leucine-rich repeat protein), another member of the SLRP-family, does not activate complement, but locally regulates the complement response by capturing the complement inhibitor C4BP (4). PRELP is an ~58-kDa extracellular protein that was originally purified as a component of bovine articular cartilage (10) but it is also found in other connective tissues such as in the sclera, tendon, skin, lung, and heart (10, 11). Belonging to the SLRP-family of proteins, PRELP contains 10 characteristic leucine-rich repeats flanked by cysteine loops in both the N and C termini. It differs from other proteins in this family by its N terminus rich in proline and arginine residues giving the protein basic properties (12). The N terminus of PRELP has been shown to bind both heparin and heparan sulfate as well as tyrosine sulfate-rich domains of extracellular matrix proteins such as fibromodulin and osteoadherin, both active in complement stimulation. PRELP can also bind to collagen via its leucine-rich repeat domain. Another ligand is perlecan, prominent in basement membranes (13). One function may be to bridge the collagen fibers in the matrix to heparan sulfate proteoglycans at the cell surface (14) and another to act as a molecular anchor between different components of the extracellular matrix.

PRELP or fragments of the protein are most likely released from the degrading cartilage in RA as are other components, even though its release or degradation by matrix proteases has not been studied in detail. Because the protein has been implicated in complement regulation and several closely related proteins have shown direct complement activating or inhibiting properties, we set out to investigate whether PRELP displays other complement modulating activities than binding C4BP.

EXPERIMENTAL PROCEDURES

Proteins and Sera—Recombinant human PRELP with a His tag was expressed from the pCEP4-BM40-hisEK vector in HEK293 cells and purified as described in Ref. 13 with minor changes. Briefly, expressed protein was collected in 4 liters of OPTI-MEM (Invitrogen), and the medium was adjusted to 0.5 M NaCl and 50 mM Tris-HCl, pH 8.0. The medium was filtered and applied to a Ni^{2+}-NTA column that was equilibrated with 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl. The column was washed with 10 volumes of equilibration buffer followed by three column volumes of 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM imidazole. The protein was eluted with 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 500 mM imidazole. PRELP-containing fractions were concentrated and dialyzed against TBS (50 mM Tris, pH 8, 150 mM NaCl). The purity of the protein was confirmed by SDS-PAGE followed by silver staining and Western blotting with a specific polyclonal rabbit antibody.

C9 was purified from human plasma according to a previously published method (15). C3b, C5, C6, C7, C8, factor B, and properdin were purchased from Complement Technologies. α-1-antitrypsin (A1AT) was purified as described in Refs. 16, 17. PRELP was labeled with ^{125}I using Iodo-Beads (Pierce). Recombinant C4BP was expressed in HEK293 cells and purified using affinity chromatography as described previously (18).

The collagen IX NC4 domain was expressed and purified as described elsewhere (15).

Normal human serum (NHS) was prepared from freshly drawn blood of eight healthy volunteers with a permit of the local ethical committee. The pooled blood was allowed to clot for 30 min at room temperature and then 1 h on ice. After two centrifugations, the serum fraction was frozen in aliquots and stored at -80 °C. To prepare heat-inactivated serum, the serum was incubated at $+56°$ for 60 min.

Patients—Synovial fluids were collected from 14 consecutive patients (4 males, 10 females) fulfilling the American College of Rheumatology criteria for rheumatoid arthritis and seeking care at the Department of Rheumatology at Lund University hospital due to a synovitis in one knee joint. The collection of synovial fluids upon informed patient consent was approved by the Regional Ethical Review Board in Lund, Sweden.

Hemolytic Assays—For the classical pathway, sheep erythrocytes were washed three times with $DGVB^{2+}$ (2.5 mM veronal buffer pH 7.35, 72 mM NaCl, 0.1% gelatin, 1 mM $MgCl_2$, 0.15 mM $CaCl_2$, 2.5% glucose) and suspended to a concentration of 10^9 cells/ml. Equal volumes of cell suspension and amboceptor (Dade Behring, diluted 1:3000 in $DGVB^{2+}$) were mixed and incubated for 20 min at $+37$ °C with gentle shaking. After two washes with $DGVB^{2+}$, 25 μl erythrocyte-suspension (5×10^8 cells/ml) was added to microtiter plate wells containing 0.1% NHS with increasing amounts of PRELP in a total volume of 150 μl. Erythrocytes suspended in water served as total lysis control. The plate was incubated in 37 °C for 60 min with shaking, followed by centrifugation at $800 \times g$ to pellet intact cells. The degree of erythrocyte lysis was determined by measuring the absorbance of the supernatant at 405 nm (Cary 50 MPR microplate reader, Varian).

For the alternative pathway, rabbit erythrocytes were washed three times with Mg^{2+}EGTA (2.5 mM veronal buffer pH 7.35, 10 mM EGTA, 7 mM $MgCl_2$, 0.1% gelatin, 2.5% glucose) and suspended to a concentration of 0.5×10^9 cells/ml. Cell-suspension (25 μl) was added to microtiter plate wells containing 1.25% NHS in Mg^{2+}EGTA with increasing amounts of PRELP. Erythrocytes suspended in water served as total lysis control. The plate was incubated at 37 °C for 60 min with shaking, followed by centrifugation at $800 \times g$ to pellet intact cells. The amount of erythrocyte lysis was determined by measuring the absorbance of the supernatant at 405 nm.

Inhibition of Complement Deposition by PRELP—Aggregated human IgG (2.5 μg/ml, classical pathway), mannan (100 μg/ml, lectin pathway) or zymosan (20 μg/ml, alternative pathway) was coated onto microtiter plates in 75 mM sodium-carbonate buffer, pH 9.6 overnight at $+4$ °C. The plates were blocked with 1% BSA in PBS for 2 h at room temperature. NHS diluted in GVB^{2+} (5 mM veronal buffer pH 7.4, 144 mM NaCl, 1 mM $MgCl_2$, 0.15 mM $CaCl_2$, and 1% gelatin) was incubated with increasing concentrations of PRELP, BSA, recombinant decorin or C4BP (classical pathway) or D(+)-mannose (lectin pathway) on ice for 30 min after which the mixtures were added to the plate. For the alternative pathway, NHS was diluted in Mg^{2+}EGTA and incubated as above with PRELP, BSA, or factor H before adding to the plate. The plates were incubated at $+37$ °C and deposited complement components were detected

with antibodies against C3d (A0063, Dako), C5 (A306, Quidel) and C9 (A226, Complement Technologies) followed by HRP conjugated secondary antibodies (P0399, P0449, Dako). The plates were developed with *o*-phenylenediamine (OPD) substrate (Dako) and H_2O_2 and the absorbance at 490 nm was measured.

Inhibition of Alternative Pathway C3-convertase—Microtiter plates were coated with 0.1% agarose in water and allowed to dry for 36 h at +37 °C. The wells were blocked with 1% BSA in PBS for 2 h at room temperature. In studies of inhibition of convertase assembly, NHS diluted in Mg^{2+}EGTA was incubated with increasing concentrations of PRELP, BSA, or factor H for 15 min on ice after which the mixture was added to the plate. The plates were incubated at +37 °C for 1 h after which deposited properdin (A239, Complement Technologies) and factor B (A235, Complement Technologies) were detected with specific antibodies followed by HRP-conjugated secondary antibodies. When studying the effect of PRELP on the dissociation of the convertase, NHS diluted in Mg^{2+}EGTA was added to the agarose-coated plate and incubated at +37 °C for 1 h. Following washing, PRELP, BSA or factor H was diluted in Mg^{2+}EGTA and added to the plate after which the plates were incubated at +37 °C for 1 h. Properdin and factor B remaining bound to the plate were then detected as described above.

Complement Deposition on PRELP—Microtiter plates were coated with 5 μg/ml PRELP in 75 mM sodium carbonate buffer, pH 9.6 overnight at +4 °C. BSA (1%) was coated as a negative control and aggregated human IgG (2 μg/ml, classical pathway) or zymosan (2 μg/ml, alternative pathway) were coated as positive controls. After blocking the wells with 1% BSA in PBS for 2 h at RT, increasing amounts of NHS diluted in GVB^{2+} (classical pathway) or Mg^{2+}EGTA (alternative pathway) were added. Deposition of C4b (Q0369, Dako) or factor B (A235, Complement Technologies) was detected with specific antibodies followed by HRP-conjugated secondary antibodies.

Direct Binding Assay—Microtiter plates were coated with complement proteins at 5 μg/ml in 75 mM sodium-carbonate buffer, pH 9.6, overnight at +4 °C. Wells coated with 5 μg/ml A1AT served as a negative control. Blocking was performed with 200 μl quench (3% fish gelatin (Nordic), 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8.0) for 2 h RT. [125]-I-labeled PRELP was diluted in binding buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 50 μg/ml BSA) and added to the plate at increasing concentrations. The plates were incubated at +37 °C for 4 h. The radioactivity remaining bound to the plate after washing was determined with a γ-counter (Wallac).

Alternatively, plates were coated with PRELP or A1AT at 10 μg/ml in the same buffer as above. After blocking with quench, heat-inactivated NHS diluted in binding buffer was added to the plates, and the plates were incubated at +37 °C for 2 h. Bound proteins were detected with specific antibodies against C5 (A306, Quidel), C7 (A308, Quidel), C8 (A225, Complement Technologies), C9 (A226, Complement Technologies) and C3d (A0063, Dako) followed by HRP-conjugated secondary antibodies (Dako). The plates were developed and measured as above.

Secretion of Properdin from Neutrophils—EDTA-plasma was separated on a Histopaque-gradient using Histopaque-1119 overlaid with Histopaque-1077 (Sigma). The sample was centrifuged at 700 × *g* for 30 min at room temperature. The granulocyte band was isolated and washed once with 0.5% human serum albumin in PBS and once with PBS, before suspending cells in RPMI containing 25 mM Hepes (HyClone) to a density of ~30 × 10^6 cells/ml. The final cell preparation contained in addition to granulocytes some contaminating erythrocytes. The cells were stimulated with 10 ng/ml GM-CSF (R&D Systems) for 0–30 min at +37 °C after which they were pelleted and 100 μl supernatant was added to Maxisorp plates coated with 10 μg/ml PRELP, A1AT or C3b. The plates were incubated for 1 h at room temperature and bound properdin was detected with a polyclonal antibody against human properdin (A239, Complement Technologies). The proportion of neutrophils in the granulocyte preparation was evaluated by staining the cells with anti-CD16 APC (21279166, Immunotools) and anti-CD4 APC (21278046, Immunotools) followed by flow cytometric analysis. The final neutrophil content of the cell preparation was up to 40%.

Inhibition of C8-deposition on Erythrocytes—Amboceptor-coated erythrocytes were prepared as described above and suspended in $DGVB^{2+}$ to a concentration of 5 × 10^8 cells/ml. Cell suspension aliquots (10 μl) were mixed with 0.1% C9-depleted serum (A326, Complement Technologies) and increasing amounts of PRELP, A1AT (negative control), or C4BP (positive control) in a total volume of 150 μl. The cells were incubated at +37 °C for 1 h with shaking after which they were pelleted and washed twice with PBS. The cells were suspended in a polyclonal antibody against C8 (A225, Complement Technologies) diluted in 1% BSA in PBS with 30 mM NaN_3 and incubated for 45 min at room temperature followed by two washes with PBS. A FITC-conjugated secondary antibody (F0250, Dako) diluted in the same buffer as above was then added to the cells and allowed to bind for 30 min at room temperature. After two washes with PBS, the cells were suspended in 50 mM Hepes pH 7.4, 100 mM NaCl, 30 mM NaN_3 and analyzed using flow cytometry.

Inhibition of C9 Incorporation into MAC—Amboceptor-coated erythrocytes were prepared as described above and suspended in $DGVB^{2+}$ to a concentration of 5 × 10^8 cells/ml. Cell suspension (25 μl) was mixed with 0.1% C9-depleted serum diluted in $DGVB^{2+}$ and incubated at +37 °C for 1 h with shaking to allow formation of C5b-8 on the erythrocyte surface. The cells were then washed twice with $DGVB^{2+}$ and suspended in 0.04 μg/ml solution of C9 (corresponding to the amount of C9 in 0.07% NHS) that had been preincubated 30 min on ice with increasing amounts of PRELP, NC4 (positive control) or A1AT (negative control). Cell lysis was allowed to proceed for 30 min at +37 °C after which the cells were pelleted, and the absorbance of the supernatant at 405 nm was measured.

C9 Polymerization on Erythrocytes—NHS (0.25%) diluted in $DGVB^{2+}$ was incubated for 30 min at +37 °C with increasing amounts of PRELP, NC4 (250 μg/ml, positive control) or A1AT (200 μg/ml, negative control). The reactions were transferred to microtiter plate wells and 30 μl of erythrocyte suspension (10^9 cells/ml, prepared as described for the classical pathway hemolytic assay) was added to each sample. After incubation for 1 h at 37 °C with shaking, the cells were washed twice with

TBS and pelleted for 10 min at 5000 rpm at 4 °C, followed by washing with 0.5 mM phosphate buffer, pH 8.0. The cell membranes were pelleted for 20 min at 15,000 rpm at 4 °C, suspended in non-reducing sample buffer and analyzed on a 2.5–10% gradient SDS-PAGE followed by Western blotting for C9 (A226, Complement Technologies). The amount of formed C9 polymer was quantified from the membrane with densitometry using the software Science Lab 2003 Image Gauge 4.1 (Fujifilm).

Detecting and Measuring PRELP in Synovial Fluid—The concentration of PRELP in synovial fluid was measured using a competition ELISA. Microtiter plates were coated with 0.2 μg/ml PRELP in 75 mM sodium-carbonate buffer, pH 9.6, overnight at +4 °C and further protein adsorption was blocked with 1% BSA in PBS for 2 h at room temperature. A polyclonal PRELP antibody (AF6447, R&D systems) was labeled with ^{125}I using the Chloramine T method. The antibody was diluted in PBS and incubated overnight at room temperature with serial dilutions of PRELP or synovial fluid in PBS. The mixtures were then added to the coated wells and incubated for 2 h at +37 °C. After washing, the amount of bound antibody was measured using a γ-counter. Synovial fluid PRELP concentrations were calculated based on the standard curve generated from the PRELP dilution series.

Western Blot of Synovial Fluid—Synovial fluids were depleted from albumin and IgG using AlbuSorb (Biotech Support Group) and protein G-Sepharose (GE Healthcare) according to instructions provided by the manufacturers. Depleted samples were concentrated by precipitation with trichloroacetic acid (TCA). Two parts sample was mixed with one part 50% (w/v) TCA and incubated for 3 h on ice. Precipitates were pelleted and washed once with ice-cold acetone. The dried pellets were dissolved in reducing SDS-PAGE sample buffer, boiled for 5 min at 95 °C, and separated on 10–15% gradient gels. Proteins were electrophoretically transferred onto polyvinylidene fluoride membranes. After blocking further direct adsorption with 3% fish gelatin in 150 mM NaCl, 0.1% Tween-20, pH 8.0 for 1 h at room temperature, the membranes were incubated with a ^{125}I-labeled polyclonal anti-PRELP antibody (AF6447, R&D Systems) diluted in blocking buffer. After washing, bound antibody was measured using a PhosphoImager (Fujifilm). The specificity of the signal was evaluated by preincubating the antibody with PRELP in solution prior to incubation with the membrane.

RESULTS

PRELP Inhibits All Three Pathways of Complement—To investigate PRELP as an inhibitor of complement, erythrocytes were subjected to complement attack in the presence of increasing amounts of PRELP and the amount of erythrocyte lysis was measured. PRELP caused a dose-dependent inhibition of lysis through the classical pathway (Fig. 1*A*), but no effect was seen on alternative pathway-induced hemolysis (Fig. 1*B*).

To document at which step the complement cascade is inhibited, microtiter plates were coated with aggregated IgG (classical pathway), mannan (lectin pathway), or zymosan (alternative pathway) as complement activating ligands and NHS preincubated with PRELP was added. Deposition of individual complement components was measured with specific antibodies.
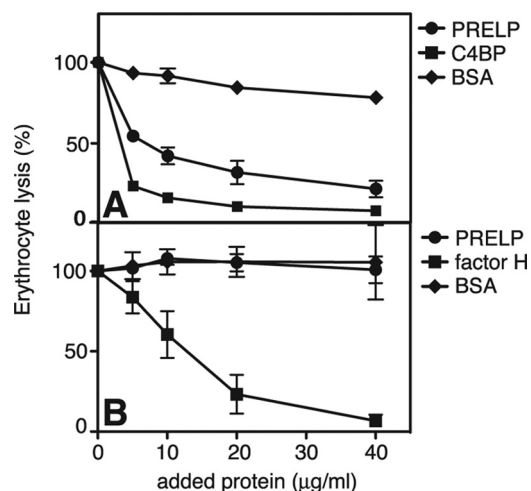

FIGURE 1. **PRELP inhibits lysis mediated by the classical pathway of complement.** To measure the inhibitory effect of PRELP on the classical pathway, antibody-coated erythrocytes were subjected to complement attack from 0.1% NHS in the presence of increasing amounts of PRELP (*A*). BSA and C4BP were used as negative and positive controls, respectively. Cell lysis was evaluated by measurement of the absorbance of the supernatant resulting from released hemoglobin. To study alternative pathway inhibition, rabbit erythrocytes were incubated with 1.25% NHS with increasing concentrations of PRELP, BSA (negative control) or factor H (positive control) (*B*). Cell lysis was measured as in *A*. The absorbance obtained without an inhibitor present was set to 100%, and the graphs show the mean and S.D. of three separate experiments.

PRELP did not inhibit deposition of C3b or C5 through the classical pathway, but strong inhibition was seen at the level of C9-deposition (Fig. 2). As a positive control we used decorin known to bind the stalks of C1q and thus inhibit the classical pathway (1), or C4BP. Similarly, PRELP did not inhibit C3b- or C5-deposition through the lectin pathway whereas that of C9 was inhibited in a dose-dependent manner (Fig. 2). D(+)mannose, which binds the heads of MBL and inhibits the binding of MBL to immobilized mannan, was used as a positive control for the lectin pathway. PRELP inhibited deposition of C3b and further C5 and C9 through the alternative pathway (Fig. 2). Therefore, it appears that PRELP can inhibit all three pathways at the level of MAC-formation and has an additional inhibitory activity on the early steps of the alternative pathway.

PRELP Inhibits the Alternative Pathway C3 Convertase—Because PRELP had the ability to inhibit deposition of C3b through the alternative pathway, we speculated that PRELP might interfere with the alternative pathway C3-convertase. Plates were coated with agarose as a complement-activating ligand and then incubated with NHS in the presence of increasing amounts of PRELP. To evaluate the amount of formed convertase, deposited properdin, and factor B were measured with specific antibodies. PRELP caused a significant decrease both in the deposition of properdin and factor B (Fig. 3*A*), even though the effect was not as strong as that of the positive control, factor H. When studying the dissociation of the convertase, NHS alone was first added to the agarose-coated plates after which PRELP was added at increasing concentration. PRELP had only a weak ability to dissociate preformed C3-convertases (Fig. 3*B*), indicating that the inhibition occurs mainly at the stage of convertase assembly.
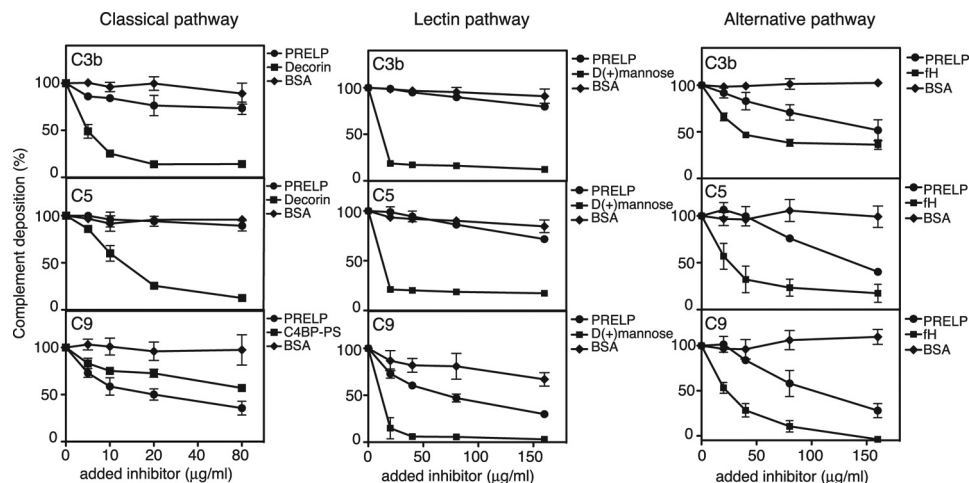
FIGURE 2. **PRELP inhibits all three pathways of complement.** To evaluate classical pathway inhibition, PRELP, decorin/C4BP (positive control) or BSA (negative control) were incubated with NHS and added to microtiter plates coated with aggregated IgG. Deposition of C3b, C5, and C9 were measured using polyclonal antibodies. For the lectin pathway, PRELP, D(+)mannose (positive control), or BSA (negative control) were incubated with NHS after which the mixtures were added to mannan-coated wells. Deposition of C3b, C5, and C9 was measured as above. For the alternative pathway, microtiter plates were coated with zymosan and NHS preincubated with PRELP, factor H (positive control), or BSA (negative control) was added. Complement deposition was measured as above. In all cases, the obtained absorbance without inhibitor was set to 100% and graphs show the mean and S.D. of three separate experiments.
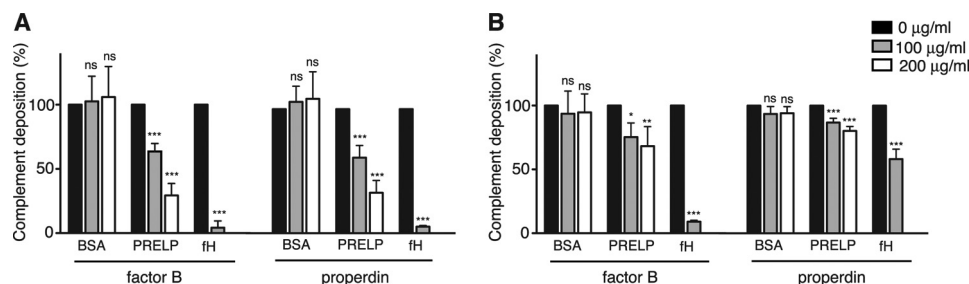
FIGURE 3. **PRELP inhibits the assembly of the alternative C3-convertase.** To study convertase assembly, microtiter plates were coated with 0.1% agarose and incubated with NHS in the presence of PRELP. Deposition of factor B and properdin was measured as an indication of formed C3-convertase (*A*). To study convertase dissociation, agarose-coated wells were first subjected to NHS alone to allow convertase formation, after which increasing amounts of PRELP was added. The amount of convertase dissociation was evaluated by measuring the amount of factor B and properdin remaining bound to the plate (*B*). The absorbance obtained without any inhibitor was set to 100%, and the graphs show the mean and S.D. of three separate experiments. Statistical significance of differences was calculated using a two-way ANOVA with a Bonferroni post-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *ns*, not significant.

PRELP Binds C3 and MAC Proteins—As PRELP interfered with the assembly and to some extent increased the dissociation of the alternative pathway C3-convertase, we hypothesized that PRELP may bind one or more of the convertase-forming proteins, *e.g.* C3b, factor B, or properdin. Furthermore, since deposition of C9 was inhibited through all three pathways, PRELP was speculated to bind components activated late in the complement cascade, C5-C9. To study possible interactions, complement proteins were immobilized, and [125]-I-labeled PRELP was allowed to bind. PRELP was found to bind properdin, C3b, C5, C7, C8, and C9 in a dose-dependent manner (Fig. 4*A*). No interaction was detected between PRELP and C6 or factor B.

PRELP was also able to capture C5, C7, C8, C9, and C3 directly from heat-inactivated serum, indicating that the interaction occurs independent of which protein is immobilized and in the presence of other serum proteins (Fig. 4*B*).

It has been reported that properdin engages in unspecific interactions upon polymerization, which is of importance when using purified properdin that might be frozen and thawed repeatedly thus enhancing polymerization (19). To investigate whether the apparent weaker interaction between PRELP and properdin is due to artifact, we studied whether PRELP can

bind properdin secreted from freshly isolated neutrophils. Granulocytes were purified on a dual Histopaque-gradient and cells were stimulated with GM-CSF for up to 30 min. Supernatants were collected and added to microtiter plates coated with PRELP, A1AT (negative control), or C3b (positive control) and bound properdin was detected with a polyclonal antibody. Properdin secreted from neutrophils was found to bind strongly to C3b but no binding to PRELP was detected, indicating that properdin secreted from neutrophils does not interact with PRELP (Fig. 4*C*). Alternatively PRELP, upon coating to the microtiter plate, acquires a conformation, which does not recognize properdin. These results suggest that the main inhibitory activity of PRELP on the assembly of the alternative C3-convertase is due to the interaction between PRELP and C3 or the forming C3-convertase itself.

PRELP Inhibits MAC Formation at the Level of C9 Deposition—Because PRELP can interact with several components of the MAC we investigated which steps of MAC formation are inhibited by PRELP. Therefore Ab-sensitized sheep erythrocytes were subjected to complement attack from C9-depleted serum in order to prevent cell lysis. Deposition of C8 was studied using flow cytometry in the pres-
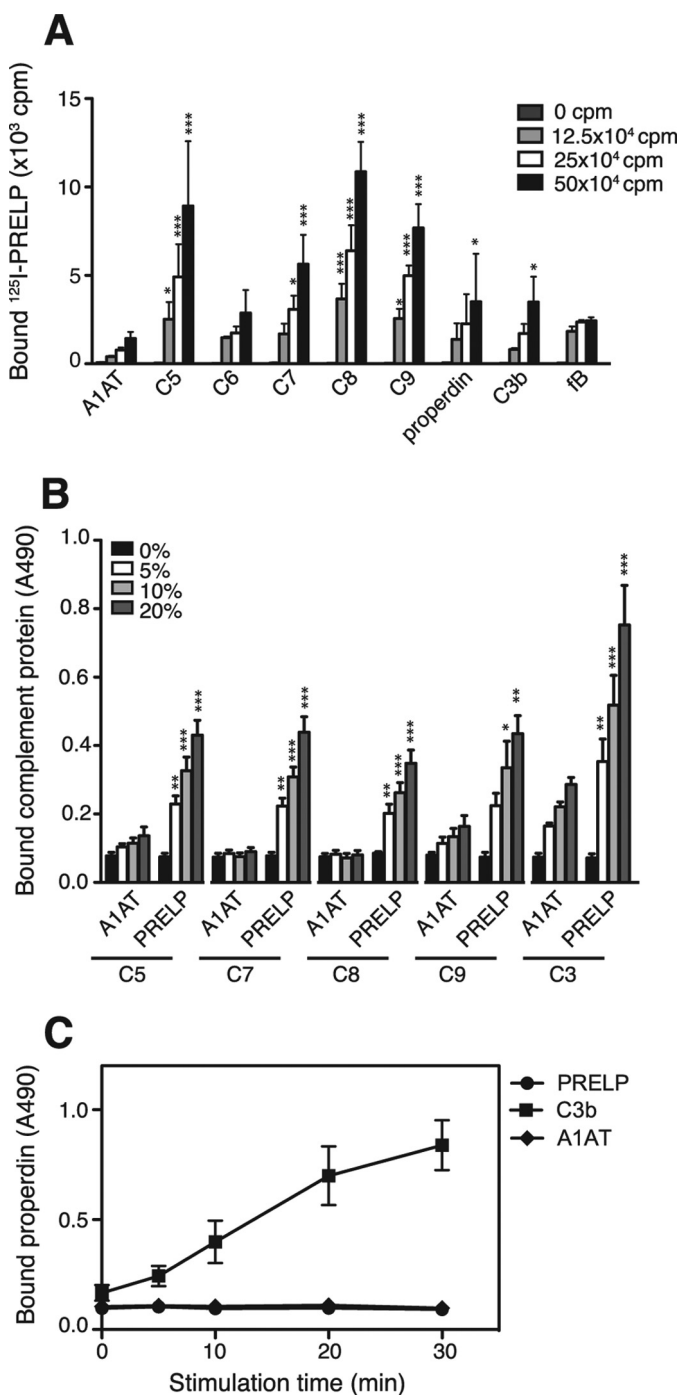
FIGURE 4. **PRELP binds MAC-components and C3.** Complement proteins were immobilized on microtiter plates and fluid-phase ^{125}I-labeled PRELP was added to the wells. Bound radioactivity was measured with a γ-counter (*A*). To study binding of PRELP to fluid-phase complement, PRELP was immobilized on microtiter plates after which heat-inactivated NHS was added at increasing concentrations (*B*). A1AT was immobilized as a negative control. Binding of properdin to PRELP was evaluated by stimulating neutrophils with GM-CSF after which cell supernatants were added to plates coated with PRELP, A1AT (negative control), or C3b (positive control). Bound properdin was measured with a polyclonal antibody (*C*). The graphs show the mean and S.D. of at least three separate experiments. Statistical significance of differences was calculated using a two-way ANOVA with a Bonferroni post-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ence of increasing amounts of PRELP. The molecule only had a weak inhibitory effect on C8-deposition compared with the positive control, C4BP (Fig. 5*A*). Therefore it

appears that PRELP mainly affects complement at the stage of C9 incorporation into MAC.

To confirm that PRELP inhibits C9 from participating in MAC formation, Ab-sensitized erythrocytes were incubated with C9-depleted serum to induce C5b8-formation on their surface. C9 that had been preincubated with increasing amounts of PRELP was then added to the cells, and lysis was evaluated by measuring hemoglobin release. As a positive control, we incubated C9 with NC4, a domain of collagen IX that has recently been shown to inhibit C9 polymerization (15), whereas A1AT was used as a negative control. PRELP inhibited erythrocyte lysis in a dose-dependent manner (Fig. 5*B*), indicating that PRELP inhibits the ability of C9 to form a functional MAC.

PRELP Inhibits C9 Polymerization—Inhibition of MAC formation by PRELP might be due to either inhibition of the interaction between C9 and C5b8 or inhibition of C9 self-polymerization. To clarify at which step the inhibitory activity takes place, erythrocytes were exposed to MAC formation in the presence of increasing concentrations of PRELP where after the erythrocyte membranes were isolated. Again, NC4 and A1AT were used as positive and negative controls, respectively. The membrane preparations were separated on a non-reducing SDS-PAGE followed by Western blotting for C9 and the level of C9 polymerization was studied using densitometric analysis of the blots. In the absence of any inhibitor, a high molecular weight band was seen on the membranes corresponding to polymerized C9 in addition to a lower molecular band representing monomeric C9 (Fig. 5, *C* and *D*). In the presence of PRELP, the high molecular weight band was absent as an indication of inhibited C9 polymerization. The monomeric band was, however, equally strong in the presence and absence of PRELP, suggesting that PRELP does not inhibit C9 from binding to C5b8, only its self-polymerization.
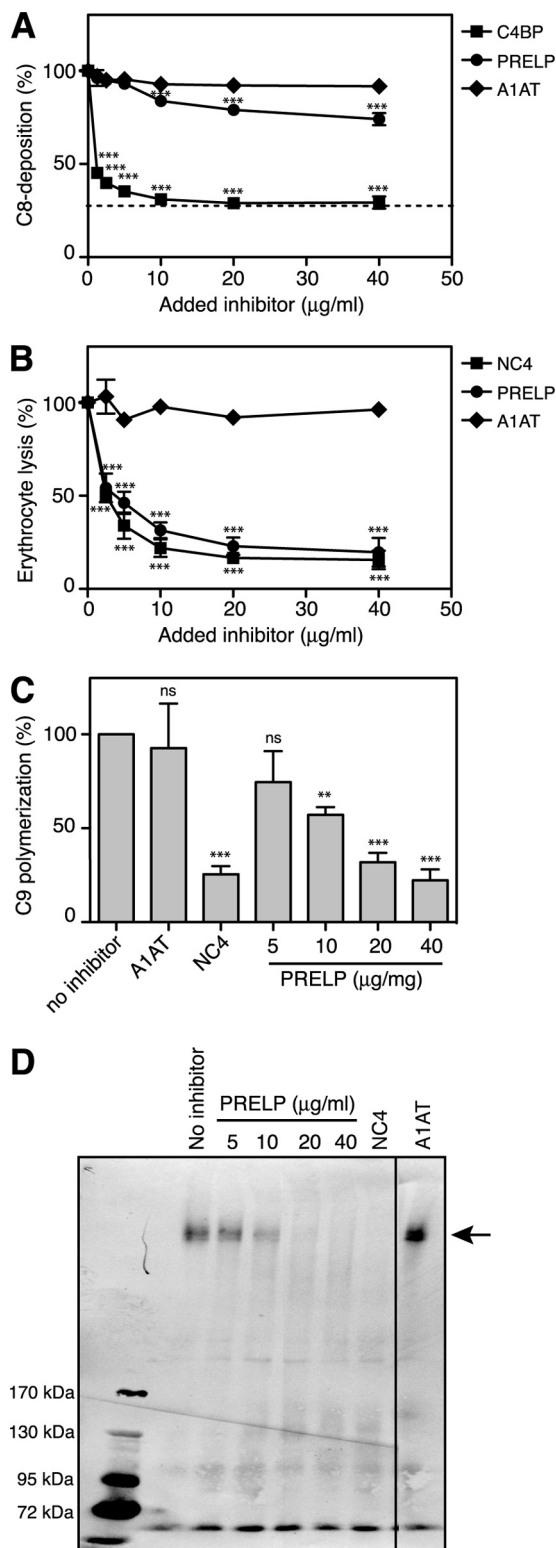
PRELP Does Not Activate Complement—To verify that PRELP is not an activator of complement, plates were coated with PRELP, alternatively IgG or zymosan as positive controls or BSA as a negative control. Increasing amounts of NHS was added to the wells and deposited C4b (classical and lectin pathway) or factor B (alternative pathway) were measured. PRELP did not induce any deposition of C4b through the classical or lectin pathway (Fig. 6*A*) or factor B through the alternative pathway (Fig. 6*B*), indicating that PRELP does not activate complement.

PRELP Is Present in Synovial Fluid—PRELP is expected to be released into the synovial fluid as a result of processes destroying the cartilage in joint disease. To verify that PRELP is present in synovial fluid we developed a competition ELISA in which synovial fluid at different dilutions or serial dilutions of PRELP were used to neutralize ^{125}-I-labeled polyclonal PRELP antibodies. The retained ability of the antibodies to bind immobilized PRELP was then measured. In a cohort of 14 RA patients, the mean synovial fluid PRELP concentration was found to be 3.5 μg/ml with a range of 1.6–7.8 μg/ml.
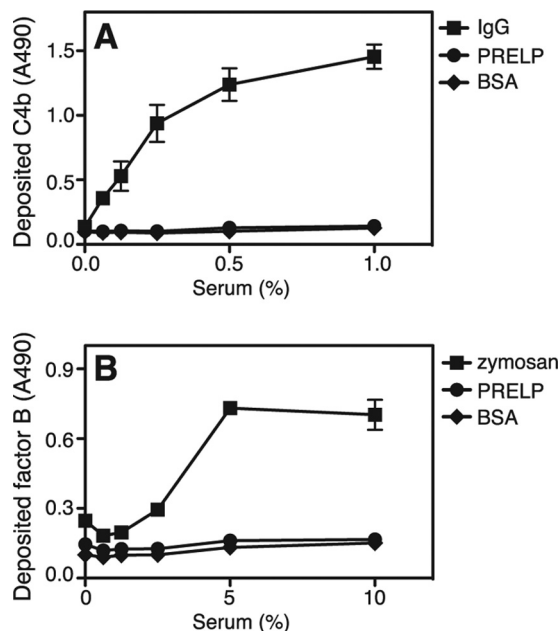
To study the nature of liberated PRELP, we performed Western blot analysis on synovial fluids from four RA patients. We found several different PRELP fragments in the patient samples, the main one being just above 50 kDa in size (Fig. 7*A*).

FIGURE 6. **PRELP does not activate complement.** Microtiter plates were coated with PRELP and NHS diluted in GVB^{2+} (classical pathway) (*A*) or Mg^{2+} EGTA (alternative pathway) (*B*) was added at increasing concentrations. BSA was coated as a negative control, whereas IgG was used as a positive control for the classical pathway and zymosan for the alternative pathway. Deposited C4b or factor B was measured as an indication of complement activation. The graphs show the mean and S.D. of three separate experiments.

Smaller fragments just above 40, 20, and 15 kDa were also found, albeit much less abundantly. A higher molecular weight band around 75 kDa was observed in all patient samples as well. The reactivity of all the bands was drastically reduced by preincubation of antibodies with PRELP protein (Fig. 7*B*). Thus, also the higher molecular weight band represents PRELP, possibly dimers or PRELP bound to other molecules and possibly a differently glycosylated PRELP.

DISCUSSION

Balance between complement activation and inhibition is essential for maintaining tissue homeostasis. In RA, complement activation is triggered by immune complexes, apoptotic/necrotic cells, and released cartilage components, which results in further activation of components of both the innate and adaptive immune system due to the interaction of complement activation products and their receptors on target cells. A number of studies have demonstrated beneficial effects of complement inhibition at the level of C3-convertase (20) or C5a generation (21, 22) in murine models of RA. Formation of MAC has

FIGURE 5. **PRELP inhibits C9-polymerization.** Amboceptor-coated sheep erythrocytes were subjected to complement attack from C9-depleted human serum in the presence of PRELP. Deposited C8 was detected by staining the cells with a polyclonal C8-antibody followed by flow cytometric analysis (*A*). C4BP and A1AT were incubated with the serum as positive and negative controls, respectively. The *dotted line* shows the background signal of the antibodies binding to cells in the absence of serum. The ability of PRELP to inhibit C9 incorporation into the MAC was studied by subjecting sensitized sheep erythrocytes to C9-depleted human serum after which C9, which was preincubated with PRELP, was added. The absorbance of the supernatant at 405 nm was measured as sign of cell lysis (*B*). A1AT was used as a

negative control whereas the collagen IX domain NC4 served as a positive control. Sheep erythrocytes were incubated with NHS in the presence of PRELP, A1AT, or NC4 after which the cell membranes were isolated and subjected to SDS-PAGE analysis followed by Western blot using a polyclonal C9 antibody. The amount of polymerized C9 on the cell surface was quantified by comparing the intensity of the high molecular weight band recognized by the C9-antibody in the different samples (*C*). A representative blot is shown in *D*. In *panels A–C*, the signal obtained in the absence of any inhibitor was set to 100%. All assays were performed on at least three separate occasions and the graphs show the mean and S.D. of three individual experiments. Statistical significance of differences was calculated with a two-way ANOVA and a Bonferroni post-test (*A* and *B*) or a one-way ANOVA with a Tukey post-test (*C*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *ns*, not significant.
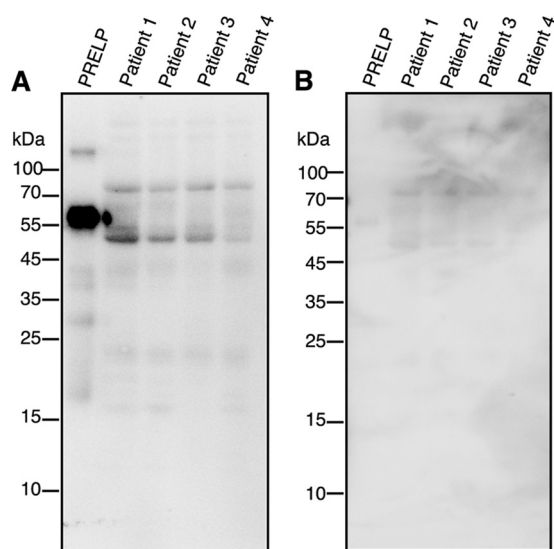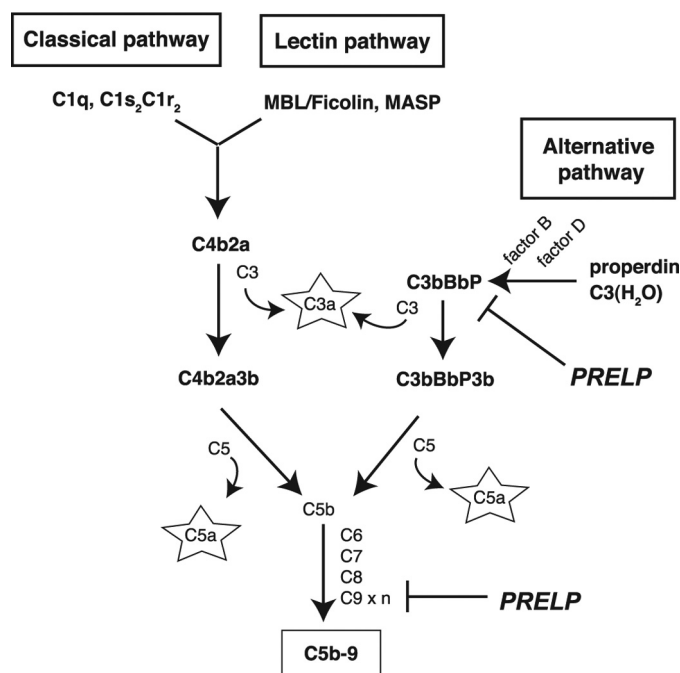
FIGURE 7. **PRELP is present in synovial fluid.** The presence of PRELP and fragments thereof in synovial fluid was analyzed by Western blot. Recombinantly expressed PRELP with a His tag has an apparent size of ~58 kDa. Bands representing several fragments of PRELP can be seen in all patient samples with the major one at a position corresponding to just above 50 kDa (*A*). The reactivity of all bands is drastically reduced by preincubating the antibody with PRELP in solution prior to incubation with the membrane (*B*).

FIGURE 8. **The complement system.** The complement system can be activated through three different pathways; the classical, the lectin, and the alternative pathway. PRELP inhibits all three pathways by interfering with C9 polymerization. PRELP furthermore inhibits the alternative pathway earlier in the cascade by inhibiting the formation of the alternative pathway C3-convertase, C3bBbP.

been shown to contribute to the pathology of the disease in mice models, as $CD59a^{-/-}$ mice develop a more severe arthritis than their wild type counterparts in an antigen-induced arthritis model (23). Furthermore, a membrane-targeted rat CD59 derivative (sCD59-APT542) was shown to decrease disease severity in the same arthritis model in rats (23, 24). Increasing our knowledge on mechanisms of complement regulation within the joints is essential for future development of specific complement inhibitors or inhibitors of the factors causing local complement activation suitable for human use with minimal systemic effects.

In the present study we have demonstrated that PRELP inhibits MAC by decreasing C9 polymerization, thereby preventing lytic pore formation. PRELP was moreover found to inhibit the alternative C3-convertase due to an interaction with C3 (Fig. 8). Inhibition of complement deposition was seen already at the level of C3 for the alternative pathway even though PRELP did not inhibit alternative pathway induced erythrocyte lysis. Because a lower serum concentration is needed for studying complement deposition than complement mediated cell lysis it makes the assay severalfold more sensitive, and the inhibitory effect observed this way would require considerably higher PRELP concentrations to be seen in the hemolytic assay. Therefore we conclude that PRELP does inhibit the alternative pathway at the C3-convertase stage as further confirmed by inhibition of deposition of properdin and factor B into the forming convertase.

PRELP has earlier been shown to bind C4BP, which might target this complement inhibitor to surfaces with degraded cartilage and exposed PRELP and thereby protect exposed surfaces from further complement deposition. Therefore it appears that PRELP has multiple targets ensuring complement inhibition as it directly targets the C3-convertase, the MAC and recruits C4BP to sites with exposed PRELP. The net outcome of PRELP-mediated complement inhibition will highly depend on the local concentration of other complement modulating molecules as well as on how PRELP is cleaved by the matrix-degrading proteases secreted by the chondrocyte or by infiltrated macrophages. In the present study, we could show that PRELP is present in the synovial fluid of RA patients, most likely released from the degrading cartilage. The PRELP concentrations varied within a range of 4 times. We showed that fragments of several different sizes of PRELP are released into the synovial fluid, while none appears to represent the intact molecule. At present there is no information on the ability of the fragments observed to inhibit complement.

Structurally PRELP resembles other members of the SLRP-family with the main sequence homology residing in the leucine-rich repeat region. The N terminus of PRELP is distinct, carrying a very cationic array of proline and arginine residues. This heparin-binding N terminus of PRELP has in previous studies been shown to have antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* similar to several other heparin-binding peptides (25). Furthermore, this domain was suggested to be a rather cell-type specific NF-κB inhibitor by binding to specific cell surface proteoglycan receptors and thereby inhibit osteoclastogenesis (14). Further investigations are, however, needed to explore the complement interacting sites on PRELP. Because MAC-inhibition has been proven beneficial in animal models of RA it would be of high interest to explore whether such intervention would ameliorate disease in patients as well. Peptide inhibitors of complement would be one solution to reduce immunogenicity of macromolecular counterparts and using allogenic proteins for this purpose would reduce possible side effects further.

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