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Complement component factor B has thrombin-like activity

Kazue Takahashi[a,*], **Nirmal K. Banda**[b], **V. Michael Holers**[b], **Elizabeth M. Van Cott**[c]

[a]Department of Pediatrics, Massachusetts General Hospital and Harvard Medical School, Boston MA 02114

[b]Division of Rheumatology, Department of Medicine, University of Colorado School of Medicine, Aurora, CO 80045

[c]Department of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114.

Abstract

Serine proteases are fundamental components of biology, including innate immunity, which is systematically orchestrated in an orderly, balanced fashion in the healthy host. Such serine proteases are found in two well-recognized pathways of an innate immune network, coagulation and complement. Both pathways, if uncontrolled due to a variety of causes, are pathogenic in numerous diseases, including coagulation disorders and infectious diseases. Previous studies have reported sequence homologies, functional similarities and interplay between these two pathways with some implications in health and disease. The current study newly reveals that complement component factor B (Bf), the second component of the alternative complement pathway, has thrombin-like activity, which is supported by a characteristic homology of the trypsin-like domain of Bf to that of thrombin. Moreover, we newly report that the trypsin-like domain of Bf is closely related to Limulus clotting factor C, the LPS sensitive clotting factor of the innate immune system. We will also discuss potential implications of our findings in diseases.

Keywords

complement factor B; thrombin; serine proteases; trypsin-like domain; MBL-associated serine protease (MASP); infectious diseases; coagulation diseases

[*]Corresponding author, Takahashi K. Current address: Department of Radiology, BUL051, Massachusetts General Hospital and Harvard Medical School, Boston MA 02114. ktakahashi1@partners.org.

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

1. Introduction

Serine proteases, fundamental molecules of biology, play a variety of key roles in the innate immune system that are tightly and intricately orchestrated to maintain a healthy status of the host. One such role is in coagulation, where a cascade of serine proteases ultimately forms a fibrin clot that is a polymerized mesh of proteolytically cleaved fragments of fibrinogen. This proteolytic activity on fibrinogen is provided by thrombin, which is also an enzymatically cleaved product of prothrombin by activated factor X (Xa) via two pathways: the intrinsic and the extrinsic pathway [1]. In a similar manner, complement component 3 (C3), the central component of the complement pathway is activated by three pathways, the classical (CP), the alternative (AP) and the lectin pathway (LP) [2]. While CP and AP are initiated by C1q and C3, respectively, LP is initiated by mannose binding lectin (MBL)-Associated Serine Protease (MASP) upon its binding to mammalian lectins, including MBL and ficolins [3,4].

Previously, we have identified that the MASP1/3 in a complex with MBL can function as thrombin, forming fibrin clots [3]. Our previous investigations also have shown that 1) Mice genetically lacking MBL or MASP1/3 have coagulopathy (prolonged bleeding time), and 2) MBL null mice develop disseminated intravascular coagulation (DIC) during bacterial infection with high mortality, compared to normal mice [4,5]. Intriguingly, thrombin and MASP1 activate protease-activated receptor 1 (PAR1), which leads to inflammatory cytokine synthesis [5].

Comparisons of molecular sequences and structural organizations have shown evolutional divergence and similarity among coagulation enzymes, complement proteins and their cascades [6–8]. Characteristic similarities are the catalytic triad formed by His (H), Asp (D) and Ser (S) [9], the oxyanion hole, and the Gly-Asp-Ser-Gly-Gly (GDSGG) motif [10]. The codon usages of the middle S in the motif have two types: tcn (n indicates a, c, g, or t), a primordial lineage and agy (y indicates c or t), a modern lineage [11]. Additionally, amino acid residue 225 of the chymotrypsin family reportedly influences serine protease activity [11,12]. Keeping these observations in mind, our study investigated whether complement factor B (Bf) has thrombin-like activity and conducted comparative analyses of its trypsin-like domain to other coagulation factors and commonality in the animal kingdom.

This report will describe and discuss our new findings that Bf shares thrombin-like activity, which is attributed to its trypsin-like domain with a sequence homology to thrombin as well as the LPS sensitive Limulus clotting factor C, the innate immune molecule of horseshoe crab [21]. The trypsin-like domain is also conserved in the animal kingdom with minor divergence. We will also discuss implications of thrombin-like activity of Bf in its potential interaction with the coagulation network with particular regard to inflammation and clinical complications.

2. Methods

2.1. Mice and reagents

Wild type (WT) C57B/6J mice were purchased from Jackson Laboratory (Acadia, MI). Bf knockout (KO) mice were previously generated [13]. Plasma was collected with ethylenediaminetetraacetic acid EDTA as an anticoagulant and stored at −80°C. Bf neutralizing monoclonal antibody was previously generated [14]. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver and Massachusetts General Hospital. Animals in both genders were used because gender has not shown effect on the results from experiments performed in this study. Purified human Bf was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Thrombin activity assay

The assays were performed as previously described [4]. Briefly, fluoresceinated thrombin substrate (R22124, Invitrogen) was mixed with diluted plasma samples with tris buffer (140 mM NaCl, 10 mM tris, pH 7.5, 10 mM $CaCl_2$). Plasma concentration in the assay was 10%. Zymosan was included at 2.5×10^6 /ml as an activator of the AP of compliment. Neutralizing mouse Bf antibodies and control IgG were used at 10%. All assays were performed in triplicates in 384 well plates. Reactions were recorded at 500 nm emission/ 520 nm excitation in a kinetic mode using SuperMax M5 plate reader (Molecular Devices). The results were expressed by Vmax (milli units/min (mU/min)) calculated by the software provided by the plate reader.

2.3. Sequence alignment

In order to analyze protein sequence homology of the trypsin-like domain of mouse Bf (mBf), human and murine coagulation factors, Limulus clotting factors and coagulation factors from other animal species were arbitrarily selected. Accession numbers are listed below. The trypsin-like serine protease domains were identified using NCBI Conserved Domain Search, www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.

Multiple protein sequences were aligned using the Crustal Omega software, www.ebi.ac.uk/Tools/msa/clustalo [15]. Amino acid numbers in alignments are from the starting amino acid, methionine, of protein translations.

Dendrogram was generated from the tree texts provided by the sequence alignments using the iTOL software, https://itol.embl.de/upload.cgi.

Human (h) protein accession numbers: Complement actor B (hB), CAA51389; Chymotrypsin (hCmTrypsin), CAA74031; Hepsin (hHepsin), AAA36013; Factor VII (hVII), AAA88040; Factor IX (hIX), CAA01607; Factor X (hX), AAA52421; Factor XI (hXI), AAA51985; Factor XII (hXII), AAB59490; Prothrombin (hThrombin), AAC63054.

Mouse (m) protein accession numbers: Complement factor B (mB), AAA63293; Chymotrypsin (mCmTrypsin), AAI03716; Hepsin (mHepsin), AAI45414; Prothrombin (mThrombin), NP_034298; Factor VII (mVII), AAH61149; Factor IX (mIX), BAE28840; Factor X (mX), EDL22110; Factor XI (mXI), AAK40233; Factor XII (mXII), CAA67891

Limulus clotting factor gene accession numbers: Limulus clotting factor C (LimC), P28175; Limulus clotting factor B (LimB), Q27081; Limulus proclotting enzyme (LimProClotE), P21902.

Bf protein accession numbers of other animal species: Lamprey (Lethenteron camtschaticum), BAA02763; Zebrafish (Danio rerio), U34662; Catfish (Channel Catfish, Ictalurus punctatus), AHH38662; Trout (Rainbow trout, Oncorhynchus mykiss), NP_001118067; Alligator (Chinese alligator, Alligator sinensis, Predicted), XM_025192933; Shark (Banded hound shark, Triakis scyllium), BAF62177; Snake (Hypsiglena sp), JAC95906; Turtle (Painted Turtle, Chrysemys picta bellii, Predicted), XM_005279607; Cattle (Bos taurus), AAI12505; Bat (Black flying fox, Pteropus alecto), ELK18904; Mouse (House mouse, Mus musculus), AAA63293; Monkey (Rhesus monkey. Macaca mulatta), AFH33517; Human (homo sapiens), CAA51389; Sea Cucumber (Apostichopus Japonicus), HQ993063; Sea urchin (Purple sea urchin, Strongylocentrotus purpuratus), NP_999700; Coral (Stony coral, Stylophora pistillata), PFX14086; Tunicate (Ascidian Tunicate, Halocybthia Roretzi), AAK00631; Sea Anemone (NematostellaVectensis), BAH22728.

3. Results

3.1. Complement factor B (Bf) shares thrombin-like activity

Plasma from wild type (WT) and Bf KO mice were assayed for thrombin-like activity using the thrombin specific fluorogenic substrate as detailed in the methods. Bf KO mouse plasma showed undetectable thrombin-like activity unlike that of WT mice (Fig 1A). Addition of mBf neutralizing antibody significantly reduced the thrombin-like activity of WT mouse plasma (Fig 1B), supporting the initial observations that the marked reduction of thrombin-like activity in Bf KO mouse plasma is due to lack of Bf. Purified human Bf showed thrombin-like activity, providing a direct evidence that Bf possesses thrombin-like activity (Fig. 1C).

3.2. Trypsin-like domain of mBf is closely related to thrombin and the Limulus clotting factor C (LimLotC)

Trypsin-like domains of Bf and coagulation factors were aligned as described in the methods section. The human and murine coagulation factors that were compared were: thrombin, which is activated by factor X, which is activated by two pathways: The intrinsic with factors XII, XI and IX in this order; and the extrinsic with factor VII in a complex with CD142 (tissue factor) [1]. Hepsin, a membrane associated type serine protease, was also included as it activates factor VII [16]. Chymotrypsin was included as a base serine protease. Three Limulus clotting factors were also included as ancestral coagulation factors.

The aligned trypsin-like domains of mBf and other coagulation factors revealed that conserved characteristic features of serine proteases were: 1) The catalytic triad of H, D and S; 2) Oxyanion hole formed with the catalytic triad with G; and 3) GDSGG motif (Fig. 2A). Codon usages of the middle S in the motif of Bf is tcn, the primordial type, which is also

observed in CmTrypsin, LimProClot, LimClotB, clotting factors XI and XII while LimClotC, thrombin, hepsin, and factors VII and X use agy, the modern type (Fig. 2A).

A dendrogram derived from the protein sequence alignment shows that Bf is closely related to LimClotC and thrombin (Fig. 2B), supporting our in vitro results that Bf shares thrombin-like activity.

3.3 Trypsin-like domain of Bf are conserved in the animal kingdom with a minor divergence

To investigate the commonality of the trypsin-like domain of Bf in the animal kingdom, we arbitrarily selected the following species: Mammals (human, monkey, mouse, cattle, bat); Avian (crow and thrush); Reptile (alligator and turtle); Bony fish (zebrafish, catfish and trout); Jawless fish (lamprey, regarded as a branching point to a vertebrae); Cartilaginous fish (shark); Urochordate (tunicate); Echinoderm (sea cucumber and sea urchin); and Cnidaria (sea anemone and coral). Accession numbers are listed in the methods section.

The protein sequence alignment of trypsin-like domains of other animals shows that the three characteristic features (the catalytic triad, the oxyanion hole, and the DGSGG motif) are well conserved with a few exceptions. In zebrafish and alligator, a minor divergence is present in the GDSGG motif, in which D is replaced with E that is the result of the third base pair change from GAt/c to GAa/g in the codon usage, respectively (Fig. 2C). For codon usage of S in the GDSGG motif, seventeen out of 20 (85%) of animals use tcn, the primordial type, whereas only three use agy, the modern type: tunicate, sea anemone and coral.

We also examined amino acid residue 730, which is Q of mBf because the amino acid, corresponding to residue 225 of chymotrypsin numbering, reportedly influences serine protease activities [11,12]. The corresponding residue is occupied with ten amino acids, depending on the species: D, I, K, L, P, Q, R, S, V, or Y. According to the study by Guinto's group [12], while the residue is dominated by Y or P, its substitution with Q or S retains serine protease activity, and other substitutions decrease activity. Bf in lamprey, shark, primates, mice, birds, and anthozoans use S, P, Q, or Y, suggesting that their Bf retain serine protease activity, possibly sharing the thrombin-like activity in these animals. In contrast, Bf from the other animals would have very low protease activity. The sequence corresponding to residue 730 is absent in zebrafish, trout and alligator, suggesting a lack of protease activity of Bf in these animals.

4. Discussion

In the current investigation, we report for the first time that Bf shares thrombin-like activity that is supported by multiple sources of evidence: Bf KO mouse plasma shows undetectable thrombin-like activity; the mBf neutralizing antibody reduces thrombin-like activity of WT mouse plasma; and native human Bf possesses thrombin-like activity. We evaluated thrombin activity using the thrombin specific fluorogenic substrate that has been successfully and widely used by others and in our previous studies, in which the activity has been correlated with fibrin clot formation, a function of thrombin [4]. The thrombin-like

activity of Bf is further supported by the sequence homology of the trypsin-like domain of Bf to that of thrombin, in which the catalytic triad, the oxyanion hole and the GDSGG motif are conserved. Thrombin-like activity of Bf has not been reported as far as we searched the literature, though its sequence homology to other domains of coagulation factors, such as von Willebrand factor has been documented by others [17]. Bf KO mice have no immunological abnormality and are fertile while AP is absent and CP is reduced due to a lack of the Bf-mediated amplification loop [18]. Taken together, these results and observations suggest that Bf can simultaneously activate AP and coagulation, as such dual activity has also been reported for MASPs [5,19].

Bf is thought to be ancestral to other complement factors as AP is also considered to be the ancient immune system [3,20]. One indicator of the ancient system is the codon usage for S “tcn”, the ancient type, in the GDSGG motif [11]. Indeed, 17 out of 20 (85%) animals compared in this study use “tcn”. Another indicator is mBf residue 730, which corresponds to residue 225 of chymotrypsin-based numbering [11]. The residue in mice and humans is occupied with Q, which retains serine protease activity, according to Guinto’s study, thus, further supporting our finding that mBf and human Bf share thrombin-like serine protease activity [12]. Interestingly, the region containing the residue is missing in alligator, trout and zebrafish. This is intriguing because coagulation factors XII and XI are also reportedly missing from zebrafish [6]. Further investigation is warranted to understand intricate interactions among coagulation and complement networks. Such complex interactions may also diverse among species and during evolution.

The phylogenic dendrogram reveals that human Bf and mBf are closely related to LimClotC, which is activated by LPS that decorates the surface of Gram-negative bacteria, such as E. coli, Salmonella and P. auruginosa [21]. In our study, we used zymosan, which is widely used to activate AP, the pathway Bf activates. Zymosan is a cell wall component of yeast, a fungus. Our findings allow us to hypothesize that upon exposure to pathogenic stimuli, Bf can behave like thrombin to trap pathogens within a fibrin clot while Bf also activates AP. Such clotting can localize pathogens and alert the host for additional microbicidal mechanisms and recruitment of phagocytes. Observations supporting this idea are that thrombin activation is protective in bacterial infection by limiting pathogen outgrowth [22]. Also, in our previous study, MBL null mice are susceptible to infection and DIC due to lack of MBL/MASP complex mediated thrombin activity [4]. Activated thrombin and MBL/MASP can recruit phagocytes, such as neutrophils and macrophages armed with microbicidal molecules and mechanisms [5,22]. Taken together, it is reasonable to hypothesize that Bf could participate by trapping pathogens locally, limiting spread, and sending danger signals to the host defense system.

Clinical complications during infection, in particular during the systemic stage, include a cytokine storm due to overwhelming systemic inflammation. One proinflammatory response mechanism is directly linked to a protease-activated receptor (PAR), in particular PAR1, which is also known as a thrombin receptor because it is activated by thrombin and also by MASP1 [5]. PAR1 is expressed on many cell types, including endothelial cells and its activation leads to intracellular signaling pathways to produce inflammatory cytokines and chemokines [5]. Bf having thrombin-like activity provides a possibility that Bf is a PAR1

activator. Detailed future study is required to determine whether Bf-mediated thrombin-like activity is efficient to activate PAR1.

Another devastating clinical complication of infection is the development of disseminated intravascular coagulation (DIC), which is associated with high mortality [23,24]. Aside from possibly the aPTT waveform [4][24], none of the tests for DIC have good specificity in predicting DIC although complement deficiencies are considered to be a risk factor [25–27]. We have previously shown that MBL null mice, which lack LP, develop DIC during bacterial infection with high mortality [4]. Hypothetically, functional CP, AP and/or LP pathways could be used to divert overwhelming thrombin activity, avoiding perturbations in a hemostasis. Under such a scenario, complement deficiency may predispose to clinical complications including DIC and cytokine storm (Fig 4).

Lastly, we propose that Bf simultaneously participates within two networks: the complement and coagulation systems. Further investigations would gain further insights into the complex and intricate balance of coagulation and complement networks in the innate immune system of healthy and disease progressing hosts.

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Highlights

- Complement factor B (Bf) has thrombin-like activity
- Trypsin-like domain of mouse Bf is homologous to those of coagulation factors of both mouse and human
- Trypsin-like domains of Bf from mouse and human are closely related to that of LPS sensitive Limulus clotting factor C
- Trypsin-like domain of Bf is conserved in the animal kingdom
- Bf involves within two networks of complement and coagulation in the immune system

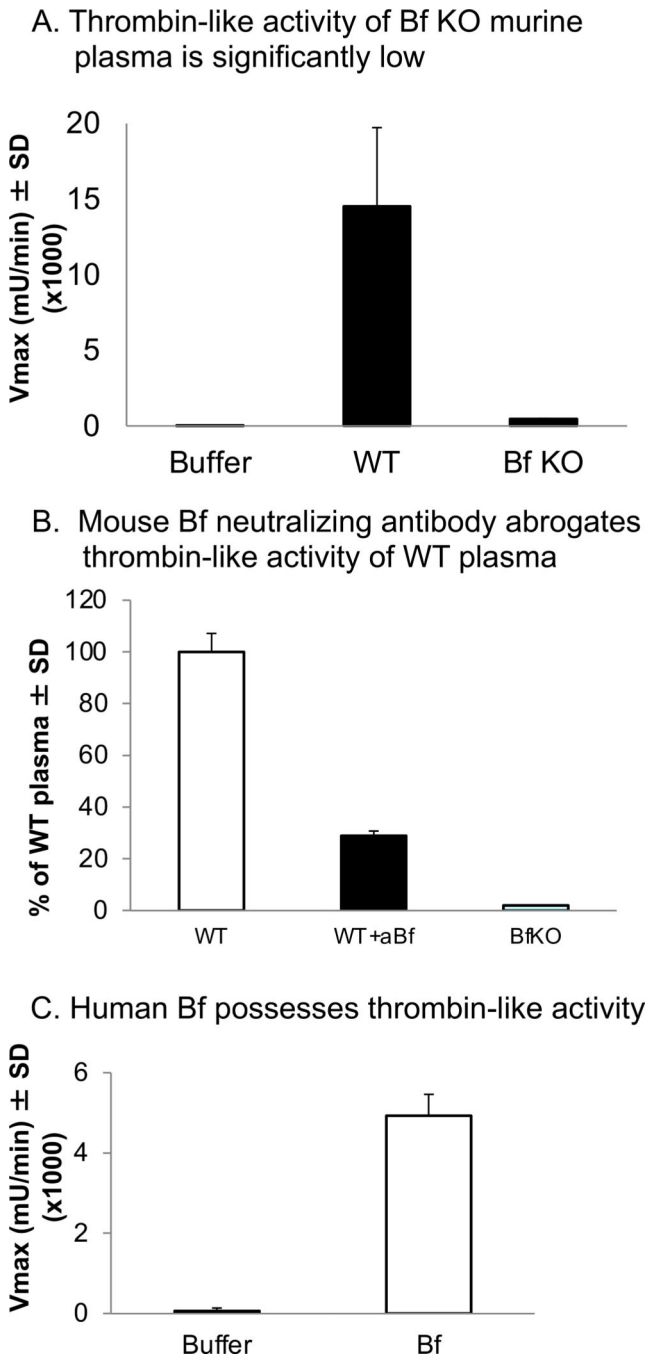

Fig. 1. Bf possesses a thrombin-like activity.

(1A) Bf KO mouse plasma showed significantly reduced thrombin-like activity compared with wild type (WT) plasma. Buffer was 140 mM NaCl, 10 mM Tris, pH 7.5, 10 mM $CaCl_2$). (1B) Addition of mouse Bf (mBf) neutralizing mAb reduced the thrombin-like activity of WT plasma. (1C) Purified human Bf showed thrombin-like activity. Assays were performed in triplicate the results were expressed as Vmax (mU/min) mean ± SD for Fig. 1A and 1C and % inhibition ± SD of WT in fig 1B.

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                            H                   D                      G S         S
mBf          512 GAVVSEYFVLTAAHCFMVD 571 FYDYDVALVKL 692 NTCKGDSGGPLIV tcn
hBf          513 GAVVSEYFVLTAAHCFTVD 572 FYDYDVALIKL 693 NTCRGDSGGPLIV tcn
LimProClot   159 GALVTNRHVITASHCVVNS 224 TYLNDIAILTL 320 DACQGDSGGPMML tcn
LimClotB     179 GSIISNKYILSAAHAFLIG 237 ENYNDIAIIEL 338 DACQGDSGGPLML tcn
mCmtrypsin    61 GSLITTSHVLTAAHCINTN 117 LLWNDIAIIKL 210 SACNGDSGGPLNC tcn
hCmtrypsin    45 GTLIASNFVLTAAHCISNT 101 LLRNDIALIKL 194 SACNGDSGGPLNC tcn
LimClotC     161 GSLLNEKWIVTAAHCVTYS 861 NLNFDIALIQL 960 DACSGDSGGPLVF agy
mThrombin    390 ASLISDRWVLTAAHCILYP 455 NLDRDIALLKL 559 DACEGDSGGPFVM agy
hThrombin    393 ASLISDRWVLTAAHCLLYP 458 NLDRDIALMKL 562 DACEGDSGGPFVM agy
mVII         221 AVLLDARWIVTAAHCFDNI 278 KINHDIALLRL 378 DACKGDSGGPHAT agy
hVII         239 GTLINTIWVVSAAHCFDKI 299 TTNHDIALLRL 399 DSCKGDSGGPHAT agy
mX           269 GTILNEFYILTAAHCLHQA 315 TYDYDIAVLRL 410 DACQGDSGGPHVT agy
hX           263 GTILSEFYILTAAHCLYQA 318 TYDFDIAVLRL 413 DACQGDSGGPHVT agy
mIX          264 GAIINEKWIVTAAHCLKPG 321 KYSHDIALLEL 415 DSCEGDSGGPHVT agy
hIX          249 GSIVNEKWIVTAAHCVETG 305 KYNHDIALLEL 400 DSCQGDSGGPHVT agy
mXII         399 GSLIAPCWVLTAAHCLQNR 439 TYQHDLALLRL 539 DACQGDSGGPLVC tcn
hXII         403 GSLIAPCWVLTAAHCLQDR 457 SYQHDLALLRL 557 DACQGDSGGPLVC tcn
mHepsin      209 GSLLSGDWVLTAAHCFPER 272 ENSNDIALVHL 366 DACQGDSGGPFVC agy
hHepsin      190 GSLLSGDWVLTAAHCFPER 253 ENSNDIALVHL 347 DACQGDSGGPFVC agy
mXI          417 GSIIGNQWILTAAHCFSGI 475 ESGYDIALLKL 568 DTCKGDSGGPLSC tcn
hXI          418 GSIIGNQWILTAAHCFYGV 476 ESGYDIALLKL 569 DACKGDSGGPLSC tcn
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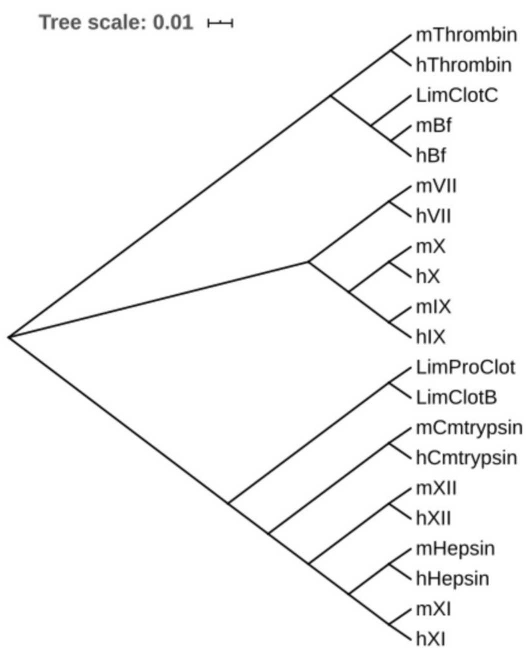

Fig. 2. Trypsin-like domains of Bf are homologous to coagulation factors of mice and humans and closely related to LimClotC.

(2A) Protein sequence alignment of the trypsin-like domain of mouse (mBf) against those of human Bf (hBf), human and murine coagulation factors, three coagulation factors of Limulus horseshoe crab, and chymotrypsin, an ancestral serine protease. Refer the methods section for protein name abbreviations. HDS, the catalytic triad is underlined at the top of alignments and forms the oxyanion binding hole along with G. GDSGG, double underlined is a characteristic motif of serine proteases. (2B) Dendrogram was generated from the alignment results in Fig 2A. Conserved amino acids are marked by asterisks below the

alignment. Colors of amino acids are assigned by the software as follows: red indicates small, hydrophobic, or aromatic; blue indicates acidic; magenta indicates basic; and green indicates hydroxyl, sulfhydryl, or amine.

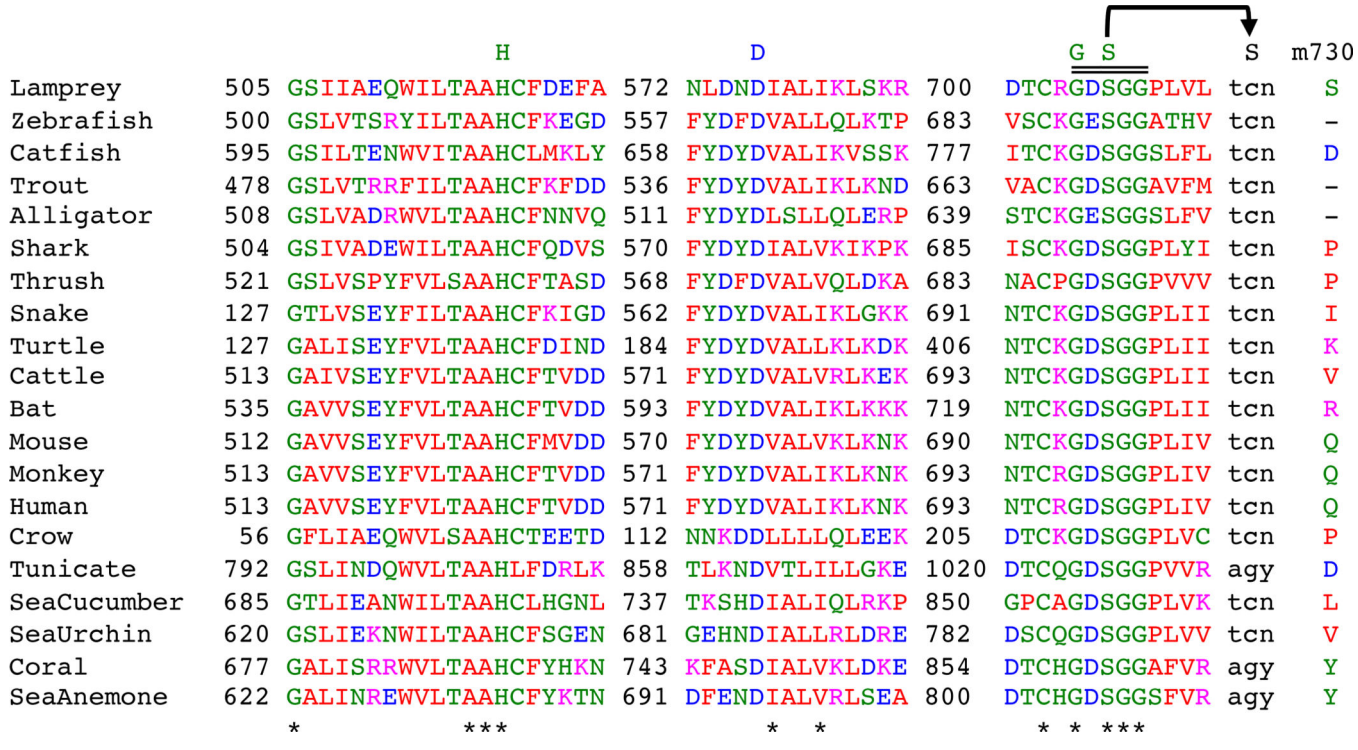

Fig. 3. Trypsin-like domain of mBf is conserved in the animal kingdom.

Protein sequence alignment of trypsin-like domains of mBf is performed against those of arbitrary selected animal species. Abbreviations for animals are found in the methods section. The description of the catalytic triad and the oxyanion biding hole are the same as in Fig 2A. Amino acids under m730 correspond to the mBf 730 residue. The asterisks below the alignment and colors of amino acids are the same as in Fig 2.

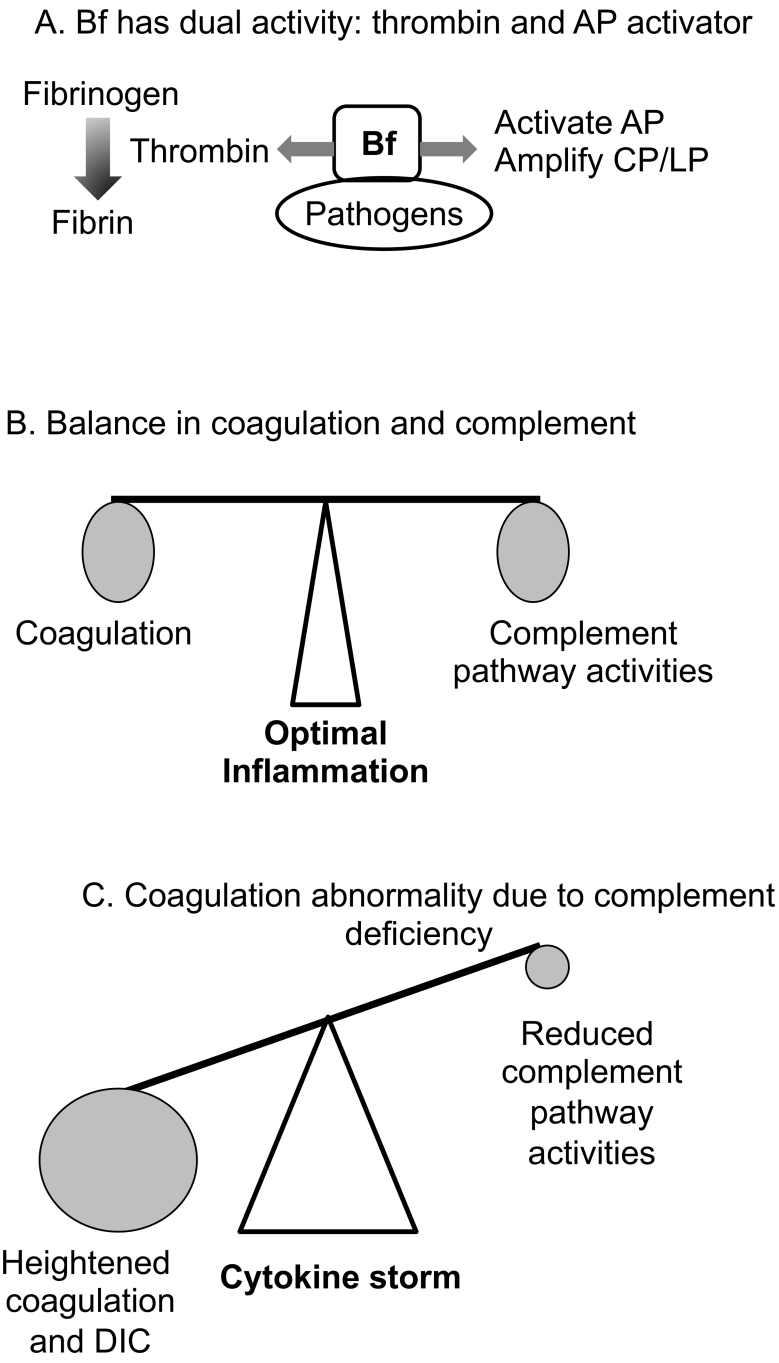

Fig. 4. Balance in coagulation and complement is important to maintain optimal host responses.

(4A) Bf has a dual activity: thrombin and AP activator upon exposure to pathogens. (4B) Reduced or impaired complement pathway activities would be unable to divert raging coagulation, resulting in a cytokine storm while complement deficiency may present as a primary problem without appropriate analyses of host conditions (4C).