# MASP-1 and MASP-2 do not activate pro-FD in resting human blood, while MASP-3 is a potential activator: kinetic analysis involving specific MASP-1 and MASP-2 inhibitors

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**Abstract**

It had been thought that complement factor D (FD) is activated at the site of synthesis and only FD lacking a propeptide is present in blood. The serum of *MASP-1/3(-/-)* mice contains pro-FD and has markedly reduced alternative pathway activity. It was suggested that MASP-1 and MASP-3 directly activate pro-FD, however other experiments contradicted this view. We decided to clarify the involvement of MASPs in pro-FD activation in normal, as opposed to deficient, human plasma and serum.

Human pro-FD containing an APPRGR propeptide was produced in insect cells. We measured its activation kinetics using purified active MASP-1, MASP-2, MASP-3 as well as thrombin. We found all these enzymes to be efficient activators, while MASP proenzymes lacked such activity. Pro-FD cleavage in serum or plasma was quantified by a novel assay using fluorescently labeled pro-FD. Labeled pro-FD was processed with half-lives of about 3 and 5 hours in serum and plasma, respectively, showing that proteolytic activity capable of activating pro-FD exists in blood even in the absence of active coagulation enzymes. Our previously developed selective MASP-1 and MASP-2 inhibitors did not reduce pro-FD activation at reasonable concentration. On the other hand, at very high concentration, the MASP-2 inhibitor, which is also a poor MASP-3 inhibitor, slowed-down the activation. When recombinant MASPs were added to plasma only MASP-3 could reduce the half-life of pro-FD.

Combining our quantitative data MASP-1 and MASP-2 can be ruled out as direct pro-FD activators in resting blood, however, active MASP-3 is a very likely physiological activator.

## Introduction

The complement system can be activated by three interconnected routes, the classical, the lectin and the alternative pathways (1). Activation of both the classical and the lectin pathways leads to the formation of the same C3 convertase, namely C4bC2a. Upon C3 cleavage C3b is generated that forms a transient complex with factor B ($FB^3$). The FB component of the pro-convertase C3bB is then cleaved by factor D (FD) to produce the alternative pathway C3 convertase, C3bBb. Hence the alternative pathway serves as an important amplification loop for both the classical and the lectin pathways, however it can be also activated on its own by the "tick-over" mechanism (1).

Components of the lectin pathway are (at least) five different pattern recognition molecules, MBL, H-ficolin (ficolin-3), L-ficolin (ficolin-2), M-ficolin (ficolin-1), and CL-LK, three MBL-associated serine proteases, MASP-1, MASP-2, and MASP-3, and two MBL-associated proteins, MAp19 (sMAP), and MAp44 (MAP-1) (2-5). MASP-1 was shown to be the exclusive initiating enzyme during the activation of the lectin pathway (6-7), and together with MASP-2, they are both essential for the C4bC2a convertase formation. On the other hand the function of MASP-3 has been unclear. MASP-1 has relatively broad substrate specificity (8) and it was implicated that it has a role in several pro-inflammatory reactions (9-10) and coagulation (11). Both MASP-1 and MASP-3 were suggested to participate in pro-FD conversion to FD (12-13) and it was speculated that even the proenzyme form of MASP-3 might be able to perform this process (13).

FD is a key enzyme for the alternative pathway. Its sole natural substrate is FB in complex with C3b (14). When purified from blood only the active form can be retrieved (15-16), and it is accepted that FD is present predominantly in the active form in normal blood (15), although some pro-FD was reported to be present in diisopropyl-fluorophosphate-treated human plasma (17). It was speculated that FD might be activated before secretion by an unknown protease within the cells (18). In most cell types, expression of natural (19) or recombinant pre-pro-FD (i.e. a construct containing a signal peptide and the putative propeptide) resulted in accumulation of the active form in the cell culture supernatant (20) although a portion of the produced FD was proenzymic (20). On the other hand pro-FD was successfully produced in insect cells (21). In vitro, trypsin was found to be the best activator of pro-FD, while thrombin, kallikrein and plasmin can also activate it with varying efficiencies (21), however, the exact kinetic constants have not been determined. In *MASP-1/3(-/-)* mice only pro-FD was found (12), and at first it was suggested that MASP-1 might be the enzyme responsible for pro-FD cleavage. However, reconstitution of the *MASP-1/3(-/-)* mouse serum with recombinant MASP-1 did not restore the alternative pathway (12). Later MASP-3 was also implicated as a possible pro-FD activator (13) suggesting a new role for this enzyme. On the

other hand alternative pathway activity was observed in *MASP-1/3(-/-)* human serum suggesting that neither MASP-1 nor MASP-3 is essential in pro-FD conversion to FD (22). Nevertheless this scientific debate brought to light some questions that for a long time have seemed to be resolved (23-24). Interestingly in *Cfh(-/-)MASP-1/3(-/-)* mice (deficient for factor H, MASP-1 and MASP-3) uncontrolled alternative pathway activation was present just like in *Cfh(-/-)* mice, suggesting that MASP-1 and -3 are not required for alternative pathway activity in *Cfh(-/-)* mice (25). In summary, the exact roles of MASPs in pro-FD activation have not been clarified yet, and additionally, we cannot exclude the possibility that significant differences may exist between human and mouse complement regarding pro-FD activation.

In this study we aimed to clarify the role of MASPs in pro-FD activation in human blood. For this purpose we determined pro-FD cleavage rates using i) purified enzymes, ii) normal human serum and plasma in the absence and presence of specific MASP inhibitors and iii) normal human plasma with exogenous MASPs added. Our quantitative data (in combination) rule out all MASP proenzymes, as well as active MASP-1 and MASP-2 as direct pro-FD activators in resting blood, however, active MASP-3 is a likely physiological activator. The term “resting blood” in this paper refers to blood, in which neither the coagulation system nor the complement system is activated above the baseline level.

## Materials and Methods

*Proteins and reagents*

Wild-type recombinant MASP-1, MASP-2, and MASP-3 catalytic fragments encompassing the CCP1-CCP2-SP domains (MASP-1cf, MASP-2cf, and MASP-3cf) were produced as described (26-27, 7). MASP-1cf and MASP-2cf are obtained as active enzymes, while MASP-3cf is produced as a zymogen. Zymogen MASP-3cf was further purified on a YMC-Q30 (YMC Europe GmbH) anion-exchange column (16 mm × 100 mm) by applying a 20-column-volume (CV) gradient of 60-200 mM NaCl in 10 mM Tris-HCl pH=8.2. Active MASP-3cf was produced from the zymogen by cleavage using MASP-1cf, and then MASP-1cf was chromatographically removed as described (28). Active MASP-3cf was further purified on a YMC-Q30 column the same way as described above for the proenzyme. High purity active MASP-3cf was eluted as the first major peak at about 150 mM NaCl. The stable zymogen variants, MASP-1cf R448Q and MASP-2cf R444Q, were prepared as described (7, 29). Human alpha-thrombin (Activity ≥2800 NIH units/mg protein, cat. no. T-1063) and bovine cationic trypsin (Activity ~10000 BAEE units/mg protein, cat. no. T-8003) were purchased from Sigma. FB and FD from human plasma were purchased from Merck (Calbiochem brand), and C3b was a gift from Prof. Mohamed R. Daha (Leiden University, Leiden, The Netherlands). The MASP-1 specific SGMI-1 and the MASP-2 specific SGMI-2 inhibitors were prepared according to Héja et al. (30). Blood was drawn from 7 healthy volunteers into Vacutainer serum tubes (BD Diagnostics) to produce fresh serum, and into Vacutainer citrate tubes (BD Diagnostics) to produce fresh plasma. After centrifugation serum samples were pooled and kept frozen in aliquots at -70 $^{o}$C to produce normal human serum. Human plasma was also prepared by centrifugation, pooled, and kept at -70 $^{o}$C in aliquots. Cy3-NHS ester and Cy5-NHS ester was purchased from GE Healthcare. Acetonitrile and ultrapure (LC-MS grade) water for mass spectrometric analysis were purchased from Merck (Darmstadt, Germany).

*Human pro-FD expression and purification*

The C-terminally poly-histidine-tagged ($His_6$) human pre-pro-FD encoding DNA was purchased as a synthetic gene from Entelechon GmbH (Regensburg, Germany). The coding sequence was manually codon optimized for expression in Sf9 cells taking into account the *Spodoptera frugiperda* codon usage frequencies found in the Codon Usage Database (http://www.kazusa.or.jp/codon/). The clone encodes the human signal peptide, the activation peptide and the mature human FD

polypeptide chain followed by a $His_6$-tag. The gene sequence and the translation are provided as a supplement (supplementary Table S1). The PstI-EcoRI fragment of the synthetic gene was subcloned into the pVL1392 (BD Biosciences) transfer vector. The resulted pVL1392_hu-pre-pro-FD-$H_6$ plasmid was used to co-transfect Sf9 cells with linearized AcNPV baculovirus DNA using the BaculoGold$^{TM}$ transfection kit (BD Biosciences). The recombinant baculovirus was amplified by several rounds, and secreted protein was verified by Western blotting with a monoclonal anti human complement FD antibody (Thermo Scientific, cat. no. GAU 010-04-02). For large scale expression of recombinant pro-FD-$H_6$ in *Spodoptera Frugiperda* Sf9 cells (BD Biosciences), a 100-mL cell suspension (cell density of $2\times10^6$ cells/mL) in Insect-Xpress protein-free medium (Lonza) was infected with the recombinant baculovirus strain by adding 1.5 mL (~$2\times10^8$ pfu/mL) of the virus stock. After 3.5 days of incubation at 27 $^oC$, the culture supernatant was collected by centrifugation (500 g, 5 min) for purification.

The recombinant human pro-FD-$H_6$ containing supernatant was gel-filtrated on a Sephadex G-25 (GE Healthcare) 50 mm × 195 mm column in 10 mM imidazole, 250 mM NaCl, pH=7.5 buffer. Fractions containing the desired protein were combined and applied to a 16 mm × 60 mm Ni-NTA Superflow (QIAGEN) column and eluted with a linear gradient of 10-250 mM imidazole, in a 250 mM NaCl, 50 mM $NaH_2PO_4$, pH=7.5 buffer. The pro-FD-$H_6$ containing fractions were combined, and dialyzed overnight excessively against 20 mM $NaH_2PO_4$, pH=6.8 at 4 $^oC$, then pro-FD-$H_6$ was applied to a 16 mm × 125 mm YMC-BioPro S30 (YMC Europe GmbH) cation-exchange column equilibrated with 20 mM $NaH_2PO_4$, pH=6.8 buffer, and eluted with a 50-350 mM NaCl linear gradient in the same buffer. During this chromatographic step two separable forms of pro-FD-$H_6$ were observed. The first eluted peak was designated as “A”, and the second peak, the major form, was designated as “B”. Fractions were assayed for purity by SDS-PAGE, and the appropriate fractions of the two forms were combined separately, then concentrated on 10-kDa cutoff concentrators to about 1 mg/mL and stored frozen in aliquots. The “B” form represents intact human pro-FD-$H_6$ (see the next section) and the term “pro-FD” will refer to this form unless otherwise stated. The concentration of pro-FD was calculated based on the extinction coefficient $\varepsilon_{280}$ = 28 460 $M^{-1}$ $cm^{-1}$, and a molecular mass of 25.8 kDa.

*Characterization of pro-FD by mass spectrometry*

Initial MALDI-TOF mass spectrometry (MS) analysis exhibited that the “B” form is consistent with the molecular weight of pro-FD-$H_6$, and the “A” form is degraded probably at the N-terminus (data not shown). The “B” form of recombinant human pro-FD was further analyzed by liquid chromatography (HPLC) coupled to electrospray (ESI) quadrupole time-of-flight (QTOF) mass

spectrometry. 10 µL of the sample was desalted and separated without pretreatment using reversed phase chromatography on a Dionex U3000 RSLC system (Thermo Scientific, Dreieich, Germany). The separation was performed on a Poroshell 300SB C18 column (1 mm × 75 mm; 5 µm particle diameter, 300 Å pore size, Agilent Technologies) at 75 $^{o}$C and 200 µL/min applying a 40-minute linear gradient with varying slopes. In detail, the gradient steps were applied as follows (minute / % Eluent B): 0/5, 3/5, 5/15, 20/32, 22/36, 29/50, 31/100, 34/100, 36/5, 40/5. Eluent B was acetonitrile with 0.1 % formic acid, and solvent A was water with 0.1% formic acid. To avoid contamination of the mass spectrometer with buffer salts, the HPLC eluate was directed into waste for the first two minutes. Continuous mass spectrometric analysis was performed using a quadrupole time-of-flight mass spectrometer (Maxis UHR-TOF, Bruker, Bremen, Germany) with an ESI source operating in positive ion mode. Spectra were taken in the mass range of 600-2000 m/z. External calibration was applied by infusion of tune mix via a syringe pump during a short time segment at the beginning of the run. Raw MS data were lock-mass corrected (at m/z 1221.9906) and further processed using Data Analysis 4.1 and MaxEnt Deconvolution software tools (Bruker, Bremen, Germany).

*Preparation of active FD from pro-FD and testing its activity*

For producing active FD from its zymogen form, pro-FD was incubated with 1/1000 amount of trypsin (by weight) at 37 $^{o}$C for 25 minutes in 20 mM $NaH_2PO_4$, ~300 mM NaCl, pH=6.8 buffer. The reaction was attenuated by 10-fold dilution with ice-cold equilibration buffer, then the sample was applied immediately to a 5 mm × 58 mm SP Sepharose High Performance (GE Healthcare) column equilibrated with 20 mM $NaH_2PO_4$, 200 mM NaCl, pH=6.8 buffer, and eluted with a 200-550 mM NaCl linear gradient in the same buffer. Fractions were assayed for purity by SDS-PAGE, then selected fractions were combined and concentrated on 10 kDa cutoff concentrators to about 0.5-1 mg/mL, then stored frozen in aliquots. The concentration of FD was calculated based on the extinction coefficient $\varepsilon_{280}$ = 28 460 $M^{-1}$ $cm^{-1}$, and a molecular mass of 25.2 kDa.

100 µg/ml C3b and 100 µg/ml Factor B were incubated with 50 µg/ml recombinant FD or plasma FD in 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, pH=7.5 at 37 $^{o}$C. Samples were taken at 0, 0.5, 1, 2, 4, 6, 24 hours. Reactions were stopped by 2-fold dilution with SDS-PAGE sample buffer and heating for 2 mins at 95 $^{o}$C. Samples were then analyzed by SDS-PAGE under reducing conditions.

*Pro-FD cleavage assay by cation exchange chromatography*

Pro-FD was dialyzed overnight excessively against 140 mM NaCl, 50 mM HEPES, 0.1 mM EDTA, pH=7.4, then concentrated to 0.5-1 mg/mL and stored frozen in aliquots. The cleavage assays were performed in the same buffer at 10 or 20 μM final pro-FD concentration, and the samples were incubated for up to 3 hours at 37 $^{o}$C. Trypsin, thrombin, active MASP-1cf, active MASP-2cf, active MASP-3cf, zymogen MASP-1cf R448Q, zymogen MASP-2cf R444Q, and zymogen MASP-3cf were applied at 0.20 nM, 635-955 nM, 33-68 nM, 13.5 nM, 30-61 nM, 7.0 μM, 6.5 μM, and 5.3-9.4 μM final concentrations, respectively, and samples were taken at every 30-40 mins. The reaction was stopped by 30-fold dilution with ice-cold equilibration buffer, then the sample was applied immediately to a 4.6 mm × 50 mm YMC-BioPro SP-F non-porous high pressure cation exchange column (YMC Europe GmbH) equilibrated with 20 mM $NaH_2PO_4$, 100 mM NaCl, pH=6.8 buffer, and eluted with a 100-450 mM NaCl linear gradient in the same buffer at 0.8 mL/min ensuring rapid analysis. The observed first order rate constant ($k_{obs}$) was determined by nonlinear regression using the $AUC = AUC_o \times \exp(-k_{obs} \times t)$ equation, where AUC is the integrated area of the pro-FD peak, and $AUC_o$ is the area at the zero time point. In order to make the rate constants of the different reactions comparable, $k_{obs}/[E]_T$ values were calculated, where $[E]_T$ is the total enzyme concentration. The $k_{obs}/[E]_T$ value can be considered as an approximation of the catalytic efficiency ($k_{cat}/K_M$) according to the Michaelis-Menten kinetics when the substrate concentration is lower then the $K_M$.

*Preparation and purification of Cy3-pro-FD and Cy5-FD*

For fluorescent labeling pro-FD and FD we used Cy3 and Cy5 CyDye monoreactive NHS Esters (GE Healthcare). Dried dyes were dissolved to 1 mg/ml in anhydrous DMF (Merck), and stored frozen in aliquots. Labeling reactions were performed in 20 mM $NaH_2PO_4$, ~300 mM NaCl, pH=6.8 buffer with 10:1 dye to protein molar ratio and were incubated in the dark for 4 hours at room temperature, then overnight at 4 $^{o}$C. The reactions were stopped by adding 1/10 volume of 1 M Tris-HCl pH=8 buffer, and after 30-fold dilution with ice-cold equilibration buffer samples were applied to a 5 mm × 55 mm YMC-BioPro S30 (YMC) column equilibrated with 20 mM $NaH_2PO_4$, pH=6.8 buffer, and eluted with a 0-350 mM NaCl linear gradient in the same buffer. Single-labeled Cy3-pro-FD had an $A_{549}/A_{280}$ ratio of about 4, and single-labeled Cy5-FD had an $A_{649}/A_{280}$ ratio of about 6. Fractions were assayed homogeneity by SDS-PAGE, then selected fractions containing single-labeled proteins were combined, then concentrated on 10 kDa cutoff concentrators to 1

mg/mL and stored frozen in aliquots. The concentrations of Cy3-pro-FD, and Cy5-FD were calculated based on the extinction coefficients of Cy3 ($\varepsilon_{549nm}$ = 150 000 $M^{-1}cm^{-1}$), and Cy5 ($\varepsilon_{649nm}$ = 250 000 $M^{-1}cm^{-1}$), and a molecular masses of 26.5 or 25.9 kDa, respectively.

*Cy3-labeled pro-FD cleavage in serum and plasma samples*

Conversion of Cy3-labeled pro-FD into Cy3-FD in serum or plasma was monitored as follows. 5 μL of 1 mg/mL Cy3-pro-FD was added to 50 μL of normal human serum or citrated plasma and the mixtures were incubated at 37 $^{o}$C for 24-26 hours. Samples were withdrawn at every hour in the first 8 hours then an overnight (24-26 h) sample was collected.

In order to test the contribution of the thrombin-content of serum to convert pro-FD into FD we added 1 mL of serum to a Vacuette Hirudin Blood Tube (Roche) containing more than 45 μg hirudin. Cy3-pro-FD conversion was tested on the same batch of hirudin-treated and untreated serum as above.

In order to test the impact of MASP inhibitors on pro-FD conversion into FD we added them to serum or plasma prior to the addition of labeled pro-FD. 1 μL of SGMI-1 (15 μM final concentration) was added to 50 μL of normal human serum or citrated plasma and the mixtures were incubated for 10 mins at room temperature, then 5 μL of 1 mg/mL Cy3-labeled pro-FD was added and the mixtures were incubated at 37 $^{o}$C for 24-26 hours. Samples were withdrawn at every hour in the first 8 hours then an overnight (24-26 h) sample was collected. 1 μL of appropriately diluted SGMI-2 (15 or 1 or 0.1 μM final concentration) was added to 50 μL of normal human serum or citrated plasma and the mixtures were incubated for 10 mins at room temperature, then 5 μL of 1 mg/mL Cy3-labeled pro-FD was added and the mixtures were incubated at 37 $^{o}$C for 24-26 hours. Samples were withdrawn at every 1-2 hours in the first 8-14 hours and finally an overnight (24-26 h) sample was collected.

In order to test the impact of exogenously provided MASPs on pro-FD conversion into FD we added 1 μL of appropriately diluted MASP-1cf, MASP-2cf or MASP-3cf (50-200 nM final concentration) to 50 μL of citrated normal human plasma and the mixtures were incubated for 10 mins at room temperature, then 5 μL of 1 mg/mL Cy3-labeled pro-FD was added and the mixtures were incubated at 37 $^{o}$C for up to 24 hours. Samples were withdrawn at every hour in the first 7 hours and finally an overnight (24-26 h) sample was collected, except when 200 nM MASP-3cf was used, where samples were withdrawn at every 15 minutes for up to 2 hours.

All reactions were stopped by 10-fold dilution with SDS-PAGE sample buffer and heating for 2 mins at 95 $^{o}$C. Cy5-labeled FD was diluted to 3.6 ng/μL with SDS-PAGE sample buffer then

treated for 2 mins at 95 $^{o}$C. 2 μL of the cleavage assay samples containing 18 ng Cy3-labeled protein were mixed with 5 μL of diluted Cy5-labeled FD (18 ng), the mixed samples were run on SDS-PAGE under reducing condition, and the gels were scanned with Typhoon (GE Healthcare) laser scanner. Band intensities were quantified by densitometry. Because the two bands were partially overlapping only the time point, where the intensity of the Cy3-pro-FD band equaled that of the Cy3-FD band, could be reliably determined on a particular gel. This time point is considered to be the half-life of Cy3-pro-FD. When the intensity of the Cy3-pro-FD band was higher at a given time point and lower in the subsequent time point than the intensity of the Cy3-FD band, we used interpolation.

## Results

*Expression and characterization of His-tagged human pro-FD*

Human pre-pro-FD was expressed in insect cells using a baculovirus vector as described in the Materials and Methods. The construct contained the human signal sequence and a C-terminal $His_6$-tag to aid purification. As previously described, insect cells produce predominantly pro-FD (21). Under our expression conditions we obtained two major variants eluting in different peaks termed "A" and "B" during the last cation exchange step. Preliminary MALDI-TOF analysis exhibited that the "B" form is consistent with the molecular weight pro-FD-$H_6$, and the "A" form is probably degraded (data not shown). Further analysis by HPLC-ESI-QTOF MS (Fig. 1) showed that the molecular weight of the "B" form is consistent with an N-terminal propeptide sequence of APPRGR. In all subsequent experiments we used the "B" form of pro-FD-$H_6$, hereafter simply termed as pro-FD. Since our major goal was to detect pro-FD activation by various serine proteases, which involves cleavage at the N-terminus, the small tag at the C-terminus most likely does not interfere with this reaction. We also converted our pro-FD into active FD by trace amounts of trypsin and purified it to remove the trypsin activity. The produced active enzyme cleaved FB-C3b in a similar fashion as the purchased control FD purified from human plasma (data not shown).

*In vitro cleavage-rates of pro-FD by various proteases*

Pro-FD can be very effectively activated by trypsin *in vitro* (21), therefore we also used bovine cationic trypsin as a highly active control protease in our assay system. Samples of pro-FD (10-20 μM) were treated with trypsin, thrombin, MASP-1cf, MASP-2cf, MASP-3cf, and also the proenzymic forms of all three MASPs at 37 $^{o}$C. The cleaving enzymes were used at concentrations suitable to produce approximately 80% consumption of pro-FD in less than 3 hours, as determined from preliminary experiments. Aliquots containing 10 μg total pro-FD + FD were withdrawn at 30-40 minute periods, diluted 30-fold with ice-cold binding buffer and analyzed immediately on a small cation exchange column as described in the Materials and Methods. The peak corresponding to pro-FD gradually disappears while a new peak with increasing intensity corresponding to FD appears over time (Fig. 2). Fitting the AUC (area under the curve) values of the pro-FD peak versus time (Fig. 3) yielded $k_{obs}$ values for each reaction. Normalized by the enzyme concentrations the cleavage efficiencies can be compared, as summarized in Table I. The highest cleavage rate, by far, is produced by trypsin, whereas active MASP-1cf, MASP-2cf, MASP-3cf, and thrombin are fairly

efficient activators of pro-FD. Proenzymic MASPs have either marginal (R448Q MASP-1cf), or no activity at all.

*Cleavage rates of pro-FD in human serum and plasma*

In order to model pro-FD cleavage in human blood we developed a novel experimental setup that is suitable to monitor pro-FD cleavage in a complex mixture. We labeled pro-FD with Cy3 dye and followed its cleavage in normal human serum or citrated plasma. In both cases Cy3-labeled pro-FD was added at 3.5 μM final concentration, and its cleavage was followed at 37 $^{o}$C. Adding the concentrated labeled protein to serum or plasma caused only a minimal (10%) dilution of the original serum or plasma samples. Aliquots were withdrawn typically at every hour, then immediately diluted 10-fold with SDS-PAGE sample buffer and heated for 2 mins at 95 $^{o}$C to stop the reactions. Portions of the treated samples containing 0.18 μL of the original serum or plasma and about 18 ng total of Cy3-pro-FD + Cy3-FD were loaded per lane and analyzed by SDS-PAGE (Fig. 4). Consumption of the Cy3-pro-FD band was apparent over time and a new band appeared with a slightly higher mobility. In most gels the SDS-PAGE samples containing Cy3-pro-FD + Cy3-FD (18 ng total) were mixed with samples containing 18 ng of Cy5-FD to allow multiplexing and unambiguous distinction of the uncleaved and cleaved forms (Fig. 4A). Our results showed that Cy3-pro-FD was converted to Cy3-FD in serum with a half-life of about 3 hours, and in plasma with a half-life of about 5 hours. The difference is probably due to thrombin and possibly other coagulation enzymes present in serum. Preliminary experiments with hirudin-treated serum revealed that the half life of Cy3-pro-FD increased by about half an hour compared to normal serum (data not shown) hence the difference between serum and plasma can only be partially attributed to the impact of thrombin.

Most importantly Cy3-pro-FD was converted into Cy3-FD in human plasma, as well, suggesting that there is a proteolytic activity capable of activating pro-FD in human blood, even in the absence of active coagulation enzymes. The activator in plasma is likely to be the physiological pro-FD activator in resting blood where activation of the coagulation cascade does not occur.

In order to assess whether any of the MASPs could be potential physiologic pro-FD activator, based on the $k_{obs}/[E]_T$ values obtained from *in vitro* cleavage experiments, we calculated corresponding MASP concentrations required to produce the observed pro-FD half-lives of about 3, and 5 hours (Table II). These data suggest that each MASP could be considered as a potential activator, but only if a significant proportion is already activated.

*Cleavage of pro-FD in human serum and plasma in the presence of MASP inhibitors*

Previously we have developed specific inhibitors for MASP-1 and MASP-2 (30). SGMI-1 is a specific MASP-1 inhibitor with a $K_i$ of about 7 nM, while SGMI-2 is a highly selective MASP-2 inhibitor with a $K_i$ of about 6 nM. We observed that SGMI-2 also inhibits MASP-3, although very poorly, with a $K_i$ of about 5 μM (6). We used these inhibitors at various concentrations to assess their effects on pro-FD activation in serum and plasma.

SGMI-1 did not affect the conversion of Cy3-pro-FD to Cy3-FD even at the highest concentration (15 μM) applied (Fig. 5 and Table III) neither in plasma nor in serum. This experiment unambiguously rules out MASP-1 as a direct pro-FD activator. Since *in vitro* active MASP-1 is a potent pro-FD activator, these results also imply that there is no significant amount of active MASP-1 in resting blood.

At 100 nM, which is well above the $K_i$ for MASP-2, SGMI-2 had no effect at all and even 1 μM of SGMI-2 had only small effect (Fig. 5 and Table III). This ruled out MASP-2 as well as direct pro-FD activator. Although we applied SGMI-2 primarily because it inhibits MASP-2, calculations presented in Table II would imply that essentially all MASP-2 would have to be in the active form to produce the measured Cy3-pro-FD half-life in plasma. This is in contrast with the observation that MASP-2 circulates as a proenzyme in the blood (32). So the results are coherent with all calculations and previous observations.

However, to our surprise, at high (15 μM) inhibitor concentration SGMI-2 significantly slowed down Cy3-pro-FD conversion both in serum and plasma (Fig. 5 and Table III). The effect was more pronounced in plasma. The difference between serum and plasma can be explained by the effect of thrombin and possibly other coagulation enzymes, which are not inhibited by SGMI-2 (6). On the other hand, the measured concentration dependence is highly consistent with the $K_i$ value of 5 μM of SGMI-2 for MASP-3 (6).

In summary, our experiments so far rule out MASP-1 and MASP-2 as physiological pro-FD activators in resting blood, suggesting that some other protease or proteases are responsible for this activity. Our observations are consistent with active MASP-3 to be such an enzyme, but also imply that for such a role significant portion of MASP-3 should be in active form even in resting blood. These assumptions require further experiments to prove unambiguously.

*Cleavage of pro-FD in human plasma in the presence exogenous MASPs*

In order to further characterize the effect MASPs on pro-FD conversion into FD we added MASP-1cf, MASP-2cf, or MASP-3cf to normal human plasma at 50-200 nM concentrations. Since we have shown before that only the active forms have considerable activity toward pro-FD (Tables I and II) we used active MASPs. At 200 nM final plasma concentration exogenously provided MASP-1 and MASP-2 did not reduce the half-life of Cy3-pro-FD at all, in spite of the fact that both can cleave pro-FD in a purified system. This suggests that both active enzymes are rapidly inactivated probably by C1-inhibitor and antithrombin. On the other hand MASP-3 reduced the half-life of Cy3-pro-FD significantly in a dose-dependent fashion (Fig 6 and Table IV). At 50 nM MASP-3cf the half-life was reduced to 2 hours, while at 200 nM to less than half an hour, suggesting that MASP-3 is not inhibited and indeed capable of cleaving pro-FD in the complex matrix of human plasma.

Summing up, the experiments in the presence of exogenously added MASPs reinforced our assumption that MASP-3 can indeed convert pro-FD into FD in plasma, and among lectin pathway proteases only MASP-3 can be considered as the pro-FD activator of resting blood.

## Discussion

Controversy in the literature regarding the role of the lectin pathway, in particular the involvement of MASP-1 and MASP-3 (12-13, 22), in the activation of the alternative pathway led us to take a closer look at the impact of MASPs on pro-FD cleavage. For this purpose we already had highly purified recombinant catalytic fragments of all MASPs, both in proenzymic and active form, and highly selective inhibitors for MASP-1 and MASP-2.

We also needed pro-FD that we produced in the baculovirus insect cell expression system. It had been previously reported that in this expression system, pro-FD is released into the culture medium in two zymogen forms with respective activation peptides AAPPRGR and APPRGR (21) in a 2:1 ratio. The high mass resolution and accuracy attained by the ESI-QTOF approach enabled us to unambiguous identify the produced proteins. In our experiments, the vast majority of pro-FD purified from insect cell culture medium contained the APPRGR activation peptide. This finding is in agreement with the predicted signal peptide cleavage site (33). Notably, contrary to what was found earlier, the other form having an additional N-terminal alanine (AAPPRGR) could be barely detected. It should be noted, however, that Yamauchi and colleagues seem to have used a shortened signal peptide (21) while we used the natural human signal peptide. So apparently APPRGR represents the dominant natural propeptide.

In order to compare their inherent capacity to activate pro-FD first we have determined the cleavage rate constants for all MASPs. Interestingly there was only a slight difference among the active MASPs indicating that, at least *in vitro*, they are all capable of activating pro-FD. The highly purified zymogen forms had zero (MASP-2 and MASP-3) or marginal (MASP-1) pro-FD cleaving activity. These results indicate that the main question is not which MASPs have the ability to cleave pro-FD, but rather which ones have sufficiently high concentration in the active form to carry out such cleavage in resting blood. On the other hand during lectin pathway activation, when all the three MASPs are present in their active form, activation of pro-FD may be accelerated considerably.

In order to model the physiologic situation, we measured the half-lives of labeled pro-FD in normal human plasma and serum. The half-life was shorter in serum indicating that coagulation enzymes, including thrombin, may be able to convert pro-FD into FD. In complement assays serum is used routinely. Our results indicate that one should be careful when drawing conclusion on the natural pro-FD activating capacity of an individual or model animal by using serum samples (23-24), as low-level activation of pro-FD might occur by backup enzymes in the serum even in the absence of the natural pro-FD activator of the resting blood. In this respect it is noteworthy that FD deficient mouse serum reconstituted to less than 1% of the normal FD level restored 23% of the

alternative pathway activity (34), suggesting that the normal level of FD is not limiting, as opposed to previous results (15).

In resting blood the coagulation cascade is not activated, and therefore plasma experiments may represent a better model for the normal physiological pro-FD conversion than serum experiments. Because labeled pro-FD was converted to FD in plasma with a half-life of about 5 hours, it is reasonable that a proteolytic activity exists in resting blood that converts pro-FD to FD. The labeled pro-FD concentration that we used was 3.5 μM, because such high concentration was necessary for reliable detection. This value is significantly higher than the physiological FD concentration (80 nM) (15), distorting unfavorably the enzyme to substrate ratio, therefore it is plausible that the pro-FD conversion rate in the circulation is higher providing a significantly shorter half-life than the measured approximately 5 hours. Another contributing factor could be the "buffer effect" of citrated plasma. It is also possible that in the presence of $Ca^{2+}$ the main converting enzyme has a higher activity. On the other hand, in plasma further activation of lectin-pathway proteases and coagulation enzymes above the baseline level can be excluded, therefore we used both serum and plasma in order to evaluate the effects of our MASP inhibitors.

When we added a MASP-1 specific inhibitor (SGMI-1, $K_i \sim 7$ nM) to serum or plasma at 15 μM final concentration the pro-FD conversion rate was no different from the inhibitor-free values. It is noteworthy that SGMI-1 inhibits both active (30) and proenzymic MASP-1 (7). Based on these observations we unambiguously ruled out MASP-1 as a direct pro-FD activator in resting blood. If MASP-1 is somehow involved in pro-FD maturation, its contribution can only be indirect e.g. through MASP-3 activation (7, 13).

To our surprise, SGMI-2, a highly selective MASP-2 inhibitor with a $K_i$ of 6 nM, did slow-down pro-FD conversion both in serum and plasma, although only at high inhibitor concentration (15 μM). The effect was more pronounced in plasma. Here we became suspicious, because according to our calculations essentially all MASP-2 would have to be in the active form to produce a 5-hour half-life for labeled pro-FD in plasma. This is in sharp contrast with previous observations that MASP-2 exists in the proenzyme form in resting blood (32). Importantly, SGMI-2 also inhibits MASP-3 (6), but only quite poorly with a $K_i$ of about 5 μM. When we examined how pro-FD cleavage rate in plasma depends on SGMI-2 concentration, we found that 1 μM SGMI-2 had a minor effect, and 100 nM SGMI-2 had no effect at all. This concentration dependence was more consistent with the involvement of an enzyme inhibited by SGMI-2 with a $K_i$ value in the micromolar range. We note that MASP-3 is such an enzyme. Nevertheless, based solely on these results, we cannot yet identify MASP-3 as the exclusive pro-FD activator in resting blood. Experiments with exogenously added MASPs further support the idea that MASP-3 is the

physiological pro-FD activator, as we demonstrated that among MASPs only MASP-3 was capable of reducing the half-life of labeled pro-FD is plasma.

Our observations support the view of Takahashi et al. (24, 35) and Iwaki et al. (13), who proposed that MASP-3 acts as a pro-FD activator. In contrast to Iwaki et al (13) who stated that even zymogen MASP-3 might activate pro-FD, we have shown that only active MASP-3 can carry out that cleavage. When Dahl and colleagues (36) first isolated MASP-3 containing complexes, the majority of MASP-3 appeared to be in the active (two-chain) form, even though they used protease inhibitors during the isolation. It is, however, not clear that activation of MASP-3 occurred during the isolation, or MASP-3 associated with MBL was already active. Our experiments suggest that for MASP-3 to be the major pro-FD activator a significant portion (at least about 10 nM) has to be in the active form in plasma to provide the observed cleavage rate. It will require further investigations to accurately determine what percentage of MASP-3 is actually present in resting blood in the active form. It is also an intriguing question how zymogen MASP-3 is activated in the absence of a lectin pathway activating surface. One possibility is that fluid phase activation of lectin pathway proteases occurs slowly at a constant rate, but active MASP-1 and MASP-2 are rapidly inactivated by their natural inhibitors like C1-inhibitor and antithrombin (37). This assumption is supported by the presence of MASP-1-C1-inhibitor and MASP-1-antithrombin complexes in normal serum and plasma (38). Importantly, as active MASP-3 is not inhibited by C1-inhibitor (39), or any other identified serpin, it could slowly accumulate in such a scenario. Our observations with exogenously added MASPs support such a mechanism. When recombinant active MASP catalytic fragments were added to plasma, only MASP-3 reduced the half-live of labeled pro-FD, indicating that while MASP-1 and -2 are inactivated rapidly MASP-3 remains active.

Taken together, we have shown that *in vitro* the active serine proteases generated during activation of the coagulation (tested with thrombin) and the complement cascades (MASP-1, -2 and -3) can cleave pro-FD with comparable efficiencies. In serum, the contribution of the coagulation proteases may be significant. Furthermore in resting blood MASP-1 and MASP-2 cannot be direct activators of pro-FD.

On the other hand, we have unambiguously demonstrated that at least one protease capable of activating pro-FD exists in normal human plasma. While further experiments are needed to unequivocally identify the physiological and perhaps exclusive pro-FD activator in resting blood, here we provided important evidences that activated MASP-3 is the likely candidate (Fig. 7).

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**Disclosures**

The authors have no conflicting financial interest.

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[3] Abbreviations used in this paper:
FB, complement factor B; FD, complement factor D; MBL, mannose-binding lectin; MASP, MBL-associated serine protease; MAp, MBL-associated protein; CCP, complement control protein domain; SP, serine protease domain; cf, catalytic fragment; Sf9, *Spodoptera frugiperda* 9 cell line; AcNPV, *Autographa californica* nuclear polyhedrosis virus; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; ESI-QTOF MS, electrospray ionization quadrupole time-of-flight mass spectrometry; $K_i$, apparent inhibition constant

## Figures

**FIGURE 1.** High resolution deconvoluted ESI-QTOF MS spectrum of recombinant pro-FD-$H_6$ in positive mode. The measured dominant mass peak (I.) was found to be in perfect agreement with the calculated mass of the intact protein with an N-terminal propeptide sequence APPRGR (25,838.15 Da). A small peak (II., Δ= -17 Da) can be assigned to succinimide formation within the same protein. A minor peak (25909.16 Da) is consistent with the AAPPRGR propeptide, but it constitutes only a minor fraction. Importantly, the analysis of the entire spectrum (10-30 kDa, see insert) clearly showed that the purified pro-FD-$H_6$ is highly homogenous and essentially free from contamination or degradation.

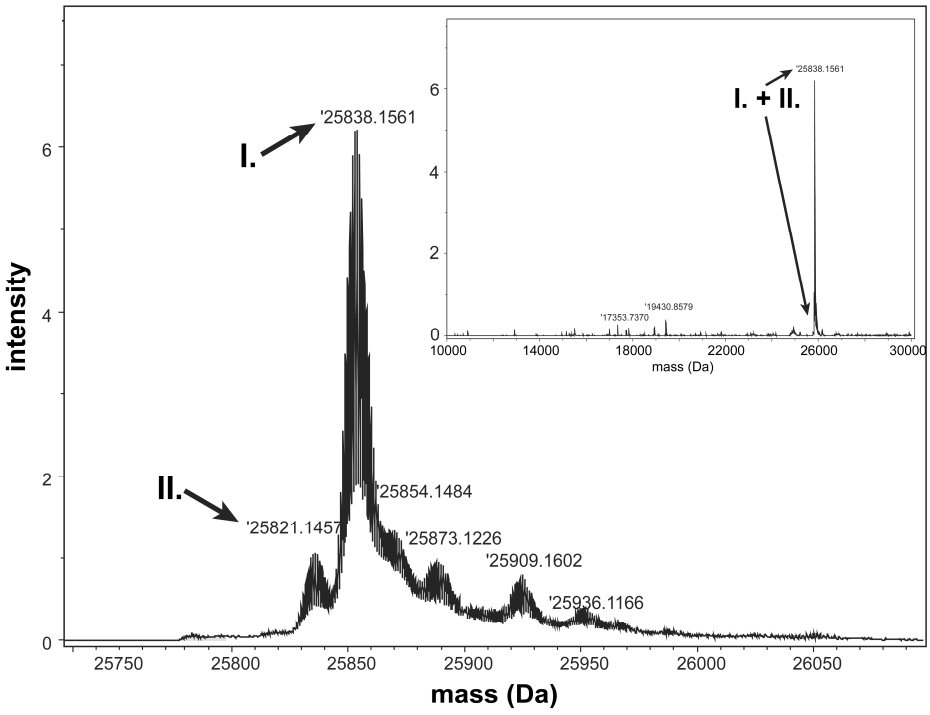

**FIGURE 2.** Detection of pro-FD cleavage by various proteases using analytical cation exchange chromatography. Typically 10 µM pro-FD was incubated in the presence of the activating proteases at 37 $^{o}$C at physiological salt concentration and pH for up to 3 hours. Samples containing a total of 10 µg pro-FD + FD were removed periodically and analyzed immediately on an analytical cation exchange column. 10 µg pro-FD (A) and 10 µg FD (B) served as controls. The cleavage by MASP-1cf (C) and MASP-3cf (D) are shown as examples. AUC (area under the curve ) values were determined by integrating the pro-FD peak, and the data were fitted as shown on Fig 3.

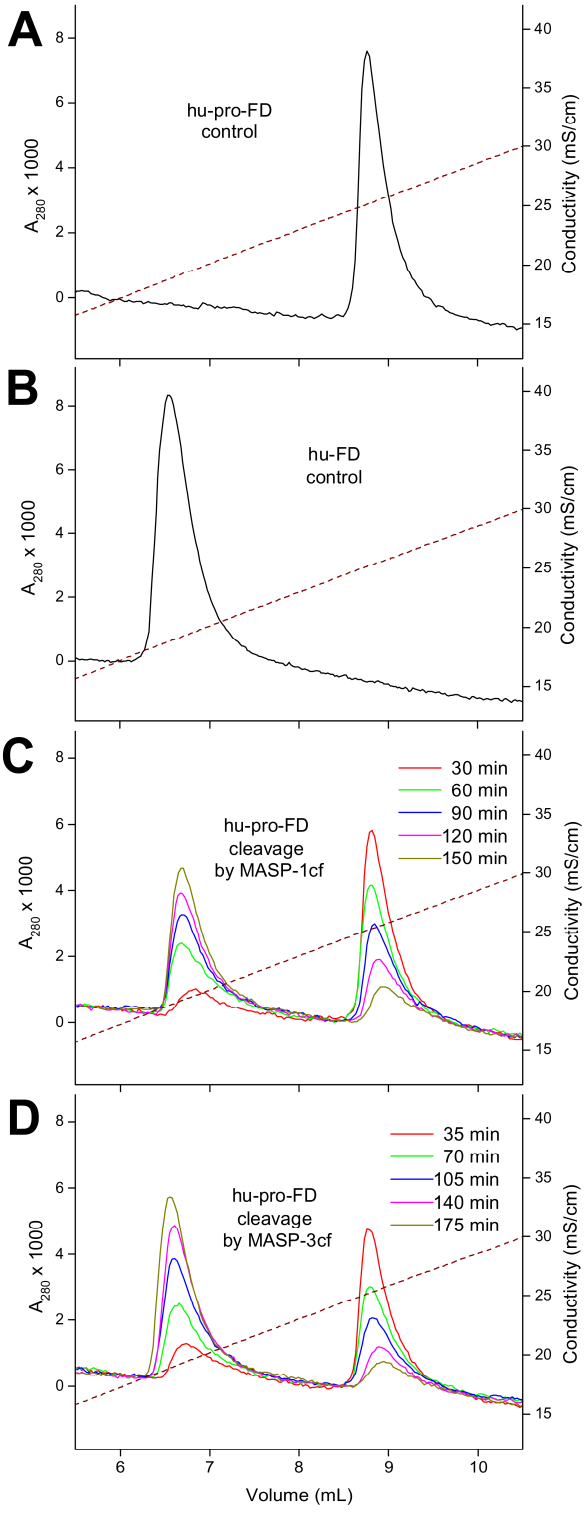

**FIGURE 3.** Fitting of the measured data to determine the cleavage rates of pro-FD by various proteases. The observed first order rate constants ($k_{obs}$) were determined by nonlinear regression using the AUC = $AUC_o \times \exp(-k_{obs} \times t)$ equation, where AUC is the integrated area of the pro-FD peak, and $AUC_o$ is the area at the zero time point (i.e. pro-FD control). The calculated $k_{obs}/[E]_T$ values are found in Table I, where $[E]_T$ is the total enzyme concentration. Data obtained from pro-FD cleavage by MASP-1cf (A) and MASP-3cf (B) are shown as examples.

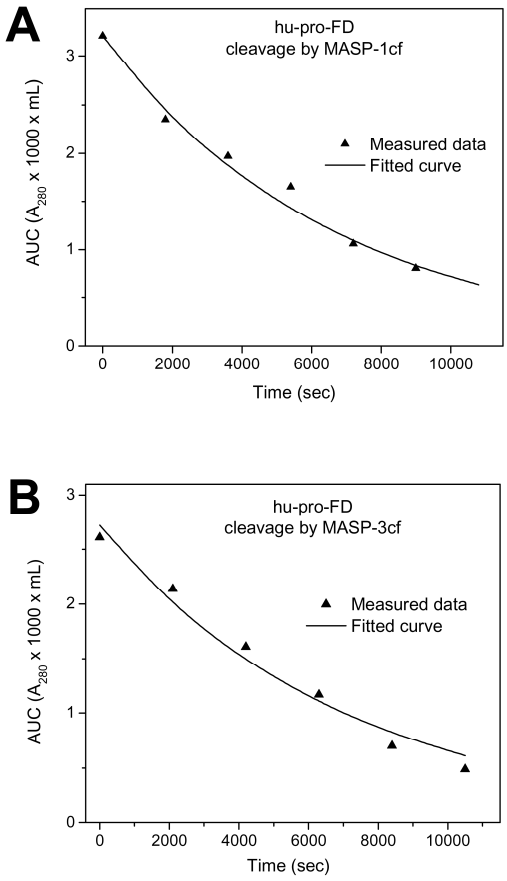

**FIGURE 4.** Determination of the half-life of Cy3-pro-FD in serum and plasma. Cy3-pro-FD was added to human serum or plasma causing only minimal dilution and the mixture was incubated at 37 $^{o}$C. SDS-PAGE was performed under reducing conditions as described in the Materials and Methods. A sample containing a total of 18 ng Cy3-pro-FD + Cy3-FD and 0.18 µL serum or plasma was applied to each lane. Because of the small difference in mobility, samples were usually multiplexed with 18 ng Cy5-FD per lane. Gels were scanned by a Typhoon laser scanner at excitation and emission wavelengths characteristic for Cy3 and Cy5 consecutively. The top panel (A) shows the overlay of the Cy3 (colored red) and Cy5 (colored green) channels for a gel with serum samples. Cy3-pro-FD disappeared over time indicating cleavage, and the produced Cy3-FD co-migrated with the Cy5-FD resulting in a yellowish-green band. The Cy3 channel of a representative gel out of 5 parallels with serum (B) and plasma (C) samples are shown on the bottom panels. The half-life of Cy3-pro-FD is considered to be the time point where the intensity of the Cy3-pro-FD band equals that of the Cy3-FD band, as explained in more detail in the Materials and methods.

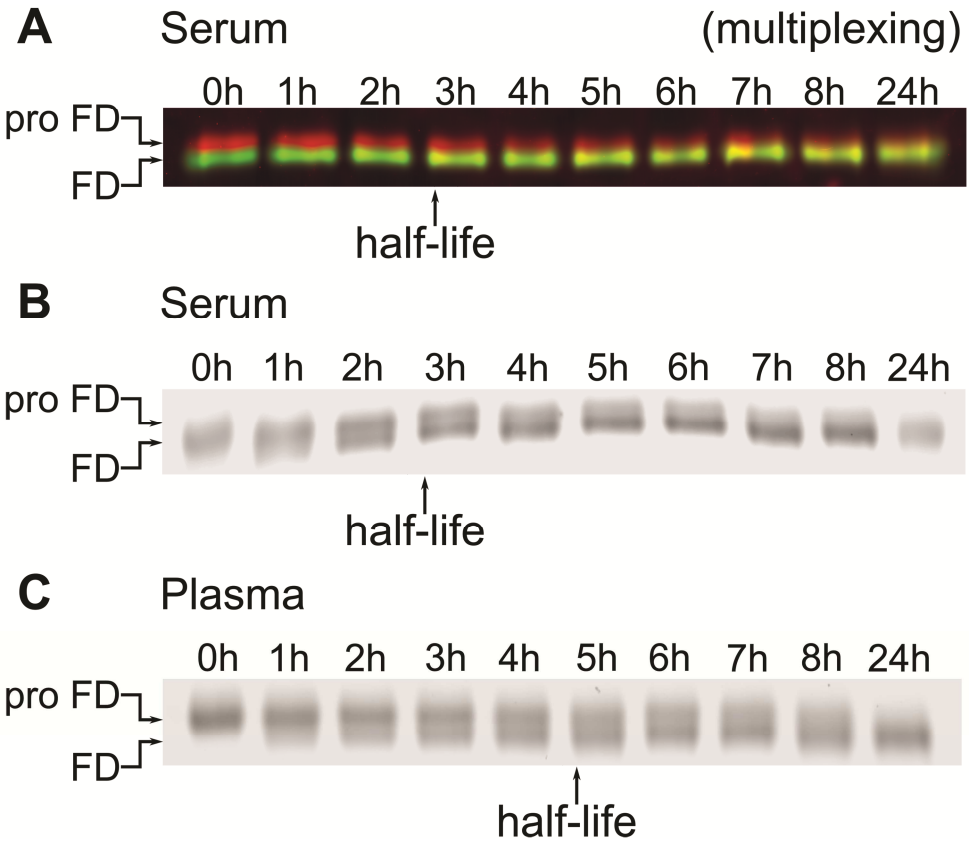

**FIGURE 5.** Determination of the half-life of Cy3-pro-FD in plasma in the presence of inhibitors. Experiments were carried out in the presence of SGMI-1, a MASP-1 specific tight-binding inhibitor (A), or SGMI-2, a tight-binding inhibitor of MASP-2, and also a poor inhibitor of MASP-3 (B and C) at various concentrations as indicated. The experiment was carried out essentially as described under Fig.4. Panels A, B, and C depict representative gels out of 2, 4, and 5 parallels, respectively.

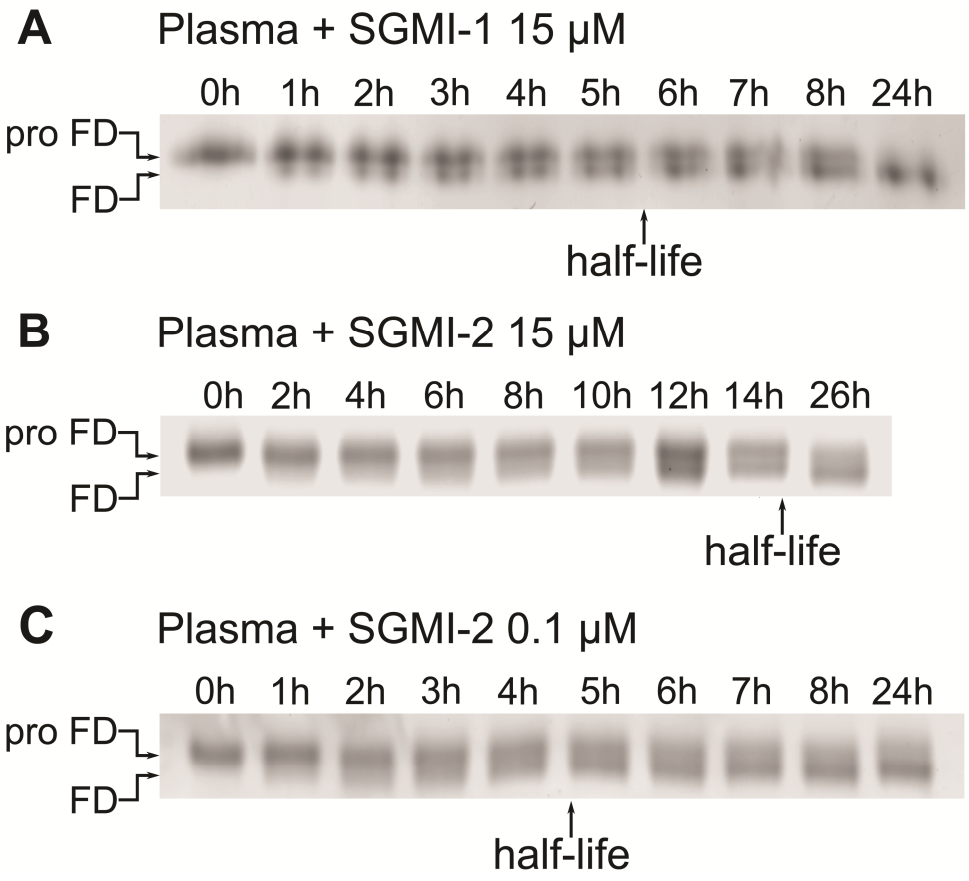

**FIGURE 6.** Determination of the half-life of Cy3-pro-FD in plasma in the presence of exogenously added MASPs. MASP-1cf (A), MASP-2cf (B) and MASP-3cf (C) were applied at 200 nM final concentration. Only MASP-3 reduced the half-life of Cy3-pro-FD compared to normal plasma. The experiment was carried out essentially as described under Fig.4. Representative gels out of 2 parallels are shown.

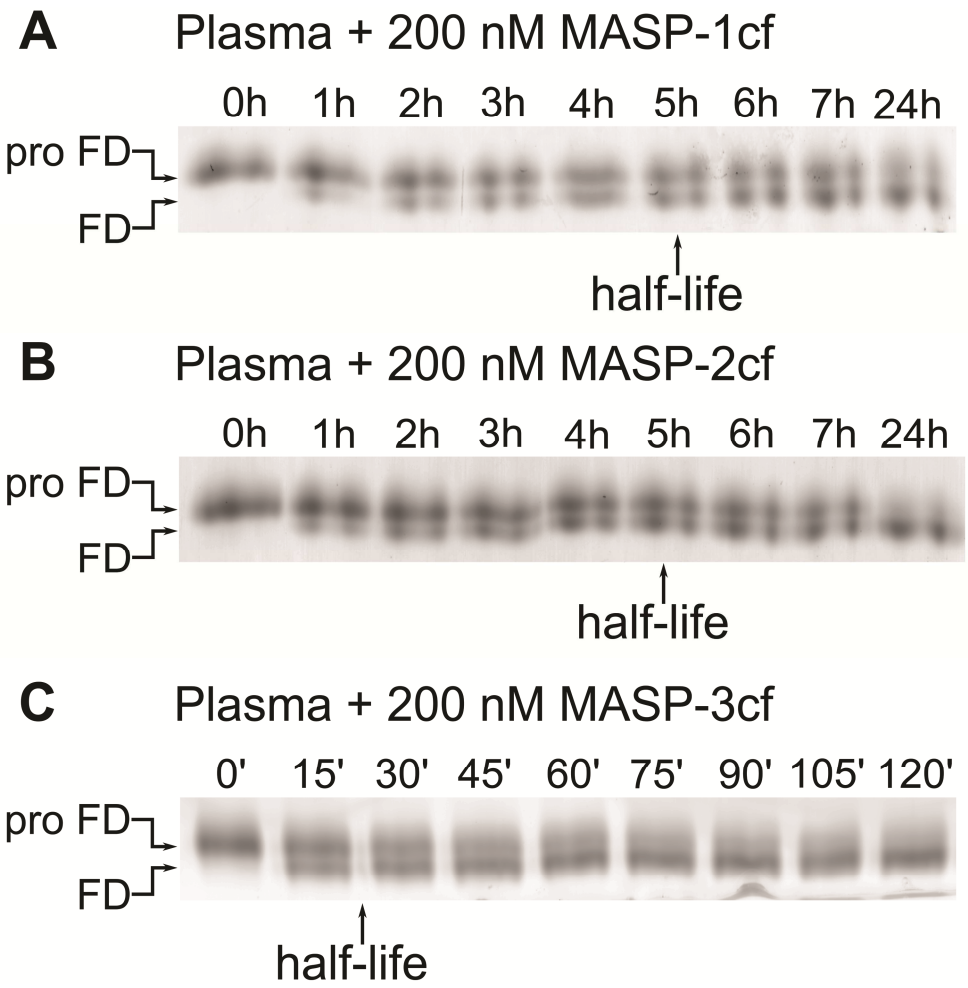

**FIGURE 7.** The suggested scenarios of pro-FD activation. The physiologically most important mechanism is the one in resting blood, because in normal blood most FD is present in the active form. In deficiencies, where a patient has mostly pro-FD, the backup mechanisms might contribute to pro-FD activation in case of injury or infection, or *in vitro* when serum is used for complement assays for testing. The most likely candidate to activate pro-FD in resting blood is active MASP-3, but further experiments are needed to unambiguously prove this assumption.

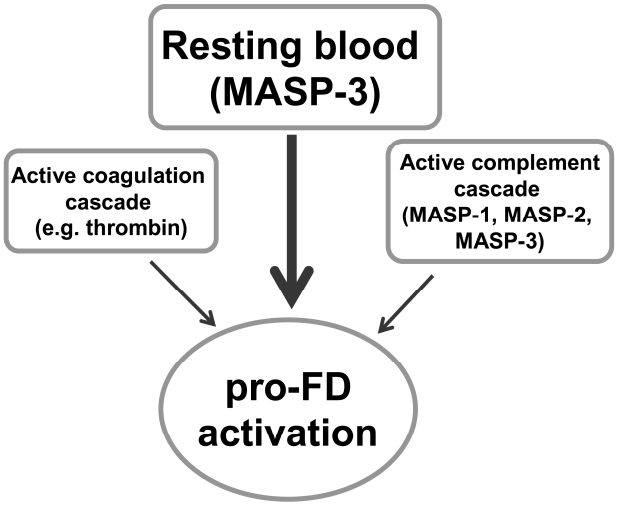

**Table I**

Activation rates of pro-FD by various proteases

| enzyme | [a] $k_{obs} / [E]_T$ ($M^{-1}$ $s^{-1}$) |
|---|---|
| bovine trypsin | [b] $1.5 \pm 0.5 \times 10^6$ |
| thrombin | [b, c] $4.6 \pm 1.2 \times 10^2$ |
| wt MASP-1cf (active) | [d] $3.9 \pm 1.2 \times 10^3$ |
| wt MASP-2cf (active) | [b] $7.2 \pm 0.4 \times 10^3$ |
| active wt MASP-3cf | [d] $4.7 \pm 1.2 \times 10^3$ |
| R448Q MASP-1cf (zymogen) | [b] $18 \pm 2$ |
| R444Q MASP-2cf (zymogen) | [e] n. d. |
| zymogen wt MASP-3cf | [e] n. d. |

[a] The $k_{obs}/[E]_T$ value can be considered as an approximation of the catalytic efficiency ($k_{cat}/K_M$) according to the Michaelis-Menten kinetics when $[S]<K_M$.
[b] Average ± range (n=2) is indicated.
[c] Obtained with thrombin thawed from frozen aliquots. Freezing may have somewhat reduced the activity of thrombin.
[d] Average ± SD (n=3) is indicated.
[e] No cleavage was detectable

**Table II**

Calculated enzyme concentrations to produce 3- and 5-hour half-lives for pro-FD

| enzyme | plasma conc. | 3 hours | | 5 hours | |
|---|---|---|---|---|---|
| | | conc. of zymogen required | conc. of active enzyme required | conc. of zymogen required | conc. of active enzyme required |
| | (nM) | (nM) | (nM) | (nM) | (nM) |
| MASP-1 | [a] 143 | 3600 | 16 | 2100 | 10 |
| MASP-2 | [a] 6 | ∞ | 9 | ∞ | 5 |
| MASP-3 | [a] 63 | ∞ | 14 | ∞ | 8 |

[a] Total concentration of all forms according to Thiel et al. (29)

**Table III**

Half-life of labeled pro-FD in serum or plasma in the presence or absence of inhibitors

| matrix | pro-FD half-life (hours) |
|---|---|
| serum | [a] 2.8 ± 0.5 |
| serum + 15 μM SGMI-1 | [b] 3.0 ± 0.5 |
| serum + 15 μM SGMI-2 | [c] 9.0 ± 1.0 |
| plasma | [a] 4.7 ± 0.5 |
| plasma + 15 μM SGMI-1 | [c] 5.5 ± 0.5 |
| plasma + 15 μM SGMI-2 | [d] 17 ± 4 |
| plasma + 1 μM SGMI-2 | [b] 6.5 ± 0.5 |
| plasma + 100 nM SGMI-2 | [a] 4.6 ± 0.5 |

[a] Average ± SD (n=5) is indicated. This 0.5 h deviation is considered to be the typical minimal error attributable the method.
[b] Two identical values were obtained, but a deviation of 0.5 h can be assumed, as explained above.
[c] Average ± range, n=2 is indicated.
[d] Average ± SD (n=4) is indicated.

**Table IV**

Half-life of labeled pro-FD in plasma in the presence or absence of exogenously added MASPs

| matrix | pro-FD half-life (hours) |
|---|---|
| plasma | [a] 4.7 ± 0.5 |
| plasma + 200 nM MASP-1cf | [b] 5.0 ± 0.5 |
| plasma + 200 nM MASP-2cf | [b] 5.0 ± 0.5 |
| plasma + 50 nM MASP-3cf | [b] 2.0 ± 0.5 |
| plasma + 200 nM MASP-3cf | [c] 0.4 ± 0.1 |

[a] Average ± SD (n=5) is indicated. This 0.5 h deviation is considered to be the typical minimal error attributable the method.
[b] Two identical values were obtained, but a deviation of 0.5 h can be assumed, as explained above.
[c] Two identical values were obtained by interpolation, but a deviation of about 0.1 h was assumed, because of the sampling intensity (every 0.25 h) near the half-life.

**Supplemental Table I**

Synthetic pre-pro-FD-$H_6$ gene sequence, translation and selected restriction sites

| Gene (and protein) name | Sequence, translation and selected restriction sites |
|---|---|
| human pre-pro-FD-$H_6$ | PstI<br>1 CTGCAGATGC ACAGCTGGGA GCGCCTGGCA GTTCTGGTCC TCCTAGGAGC<br>M H S W E R L A V L V L L G A<br><br>51 GGCCGCCTGC GCGGCGCCGC CCCGTGGTCG GATCCTGGGC GGCAGAGAGG<br>A A C A A P P R G R I L G G R E A<br><br>101 CCGAGGCGCA CGCGCGTCCC TACATGGCGT CGGTGCAGCT GAACGGCGCG<br>E A H A R P Y M A S V Q L N G A<br><br>151 CACCTGTGCG GCGGCGTCCT GGTGGCGGAG CAGTGGGTGC TGAGCGCGGC<br>H L C G G V L V A E Q W V L S A A<br><br>201 GCACTGCCTG GAGGACGCGG CCGACGGCAA GGTGCAGGTT CTCCTGGGCG<br>H C L E D A A D G K V Q V L L G A<br><br>251 CGCACTCCCT GTCGCAGCCG GAGCCCTCCA AGCGCCTGTA CGACGTGCTC<br>H S L S Q P E P S K R L Y D V L<br><br>301 CGCGCAGTGC CCCACCCGGA CAGCCAGCCC GACACCATCG ACCACGACCT<br>R A V P H P D S Q P D T I D H D L<br><br>351 CCTGCTGCTA CAGCTGTCGG AGAAGGCCAC ACTGGGCCCT GCTGTGCGCC<br>L L L Q L S E K A T L G P A V R P<br><br>401 CCCTGCCCTG GCAGCGCGTG GACCGCGACG TGGCACCGGG AACTCTCTGC<br>L P W Q R V D R D V A P G T L C<br><br>451 GACGTGGCCG GCTGGGGCAT AGTCAACCAC GCGGGCCGCC GCCCGGACAG<br>D V A G W G I V N H A G R R P D S<br><br>501 CCTCCAGCAC GTGCTCTTGC CGGTGCTGGA CCGCGCCACC TGCAACCGTC<br>L Q H V L L P V L D R A T C N R R<br><br>551 GCACGCACCA CGACGGCGCC ATCACCGAGC GCTTGATGTG CGCGGAGAGC<br>T H H D G A I T E R L M C A E S<br><br>601 AATCGCCGTG ACAGCTGCAA GGGTGACTCC GGCGGCCCGC TGGTGTGCGG<br>N R R D S C K G D S G G P L V C G<br><br>651 CGGCGTGCTC GAGGGCGTGG TCACCTCGGG CTCGCGCGTT TGCGGCAACC<br>G V L E G V V T S G S R V C G N R<br><br>701 GCAAGAAGCC CGGCATCTAC ACCCGCGTGG CGAGCTATGC GGCCTGGATC<br>K K P G I Y T R V A S Y A A W I<br><br>EcoRI<br>751 GACAGCGTCC TGGCCCATCA TCATCATCAT CATTAAGAAT TC<br>D S V L A H H H H H H * |

Only the restriction sites used for subcloning are shown (underlined)