Kinetic Analysis of a Unique Direct Prothrombinase, *fgl2*, and Identification of a Serine Residue Critical for the Prothrombinase Activity[1]

Camie W. Y. Chan,* Matthew W. C. Chan,* Mingfeng Liu,* Laisum Fung,* Edward H. Cole,* Julian L. Leibowitz,† Philip A. Marsden,* David A. Clark,*‡ and Gary A. Levy[2]*

***fgl2* prothrombinase, by its ability to generate thrombin, has been shown to be pivotal to the pathogenesis of viral-induced hepatitis, cytokine-induced fetal loss syndrome, and xeno- and allograft rejection. In this study, the molecular basis of *fgl2* prothrombinase activity was examined in detail. Purified *fgl2* protein generated in a baculovirus expression system had no measurable prothrombinase activity, whereas the activity was restored when the purified protein was reconstituted into phosphatidyl-L-serine-containing vesicles. Reconstituted *fgl2* catalyzed the cleavage of human prothrombin to thrombin with kinetics consistent with a first order reaction, with an apparent V_{max} value of 6 mol/min/mol *fgl2* and an apparent K_m value for prothrombin of 8.3 μM. The catalytic activity was totally dependent on calcium, and factor Va (500 nM) enhanced the catalytic efficiency of *fgl2* by increasing the apparent V_{max} value to 3670 mol/min/mol *fgl2* and decreasing the apparent K_m value for prothrombin to 7.2 μM. By a combination of site-directed mutagenesis and production of truncated proteins, it was clearly shown that residue Ser^{89} was critical for the prothrombinase activity of *fgl2*. Furthermore, *fgl2* prothrombinase activity was not inhibited by antithrombin III, soybean trypsin inhibitor, 4-aminobenzamidine, aprotinin, or phenylmethylsulfonyl fluoride, whereas diisopropylfluorophosphate completely abrogated the activity. In this work we provide direct evidence that *fgl2* cleaves prothrombin to thrombin consistent with serine protease activity and requires calcium, phospholipids, and factor Va for its full activity.** ***The Journal of Immunology*, 2002, 168: 5170–5177.**

Coagulation proteins play important roles in the pathogenesis of human diseases including virus-induced hepatitis (1, 2), atherosclerosis (3, 4), systemic lupus encephalitis (5), septic shock (6), and cancer (7). The generation of thrombin is a crucial reaction in blood coagulation and hemostasis. Thrombin is formed from its inactive zymogen, prothrombin, under physiological conditions, by the action of a prothrombinase complex composed of the serine protease factor Xa, with calcium, a negatively charged phospholipid surface, and the nonenzymatic cofactor, factor Va (8). The serine protease, factor Xa, is the classically known physiological activator of prothrombin. Factor Xa is activated from its zymogen precursor factor X in the presence of calcium by complexes in the intrinsic (factors IXa/VIIIa) (9) and extrinsic (factor VIIa/tissue factor) (10) pathways.

Our laboratory has previously demonstrated the pivotal role of a unique direct prothrombinase, fgl2, in the pathogenesis of fulminant viral hepatitis secondary to murine hepatitis virus strain-3 (MHV-3)[3] infection (11–13). The gene *fgl2* was originally cloned from CTLs, and the encoded protein was named as a fibrinogen-like protein due to homology to the carboxyl terminus of the β- and γ-chains of fibrinogen (14). Several lines of evidence indicate fgl2 exhibits coagulation activity. First, following MHV-3 infection, mRNA transcripts of *fgl2* and *fgl2* protein can be detected predominantly in liver reticuloendothelial cells, followed by fibrin deposition and widespread hepatic necrosis (13). We have recently reported a similar relationship of fgl2 to fulminant hepatitis B virus infection (1). Second, neutralizing mAb to fgl2 prevents hepatic fibrin deposition, liver cell necrosis, and the lethality of MHV-3 infection (15). Third, fgl2 is also expressed in a model of Th1 cytokine-induced fetal loss syndrome in which fibrin deposition is a prominent feature (16, 17), and neutralizing Ab to fgl2 prevents cytokine-induced fetal loss (16). Recently, Ruegg and colleagues (18, 19) have reported the expression of FGL2 in human peripheral blood T lymphocytes and termed the encoded protein fibroleukin. Interestingly, FGL2 expressed by T cells as a secreted form lacks a coagulation activity, and the function for T cell generated fgl2/fibroleukin remains undefined.

Based on our previous findings, we believe induction of *fgl2* represents a novel mechanism whereby macrophages and endothelial cells activate the coagulation cascade. However, the nature of this *fgl2* coagulation activity has not been fully defined. In this paper, we generated murine *fgl2* protein by a baculovirus expression system and examined the molecular basis of its prothrombinase activity. Using purified *fgl2*, we assessed the kinetic parameters of thrombin generation. We further demonstrated that phospholipids, calcium, and factor Va were required for the optimal catalytic activity of *fgl2*. A combination of site-directed mutagenesis and production of truncated proteins identified a domain and a single amino acid residue essential for the coagulation activity of *fgl2*.

*Multi Organ Transplant Program, Toronto General Hospital and University of Toronto, Toronto, Ontario, Canada; †Department of Pathology, Texas A&M University, College Station, TX 77843; and ‡McMaster University, Hamilton, Ontario, Canada

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[2] Address correspondence and reprint requests to Dr. Gary A. Levy, Multi Organ Transplant Program, Toronto Hospital, 621 University Avenue, 10th Floor, Room 116, Toronto, Ontario M5G 2C4, Canada. E-mail address: gl*fgl2*@attglobal.net

[3] Abbreviations used in this paper: MHV-3, murine hepatitis virus strain-3; Sf9, *Spodoptera frugiperda* ovarian cell 9; H5, High Five; CHO, Chinese hamster ovary; PS, phosphatidyl-L-serine; PC, phosphatidylcholine; DFP, diisopropylfluorophosphate; Ni-NTA, nickel-nitrilotriacetic acid.

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Materials and Methods

Cell culture

Log-phase *Spodoptera frugiperda* ovarian cells 9 (Sf9) and High Five (H5) cells (Invitrogen, Carlsbad, CA) were maintained in Insect Pathology Laboratory 41 medium supplemented with 10% FBS, 10 μg/ml gentamicin, 3.3 μg/L yeastolate, 2.5 μg/ml fungizone, and 1× lipid concentrate at 27°C as monolayers. Recombinant baculovirus carrying murine fgl2 cDNA was generated in Sf9 cells and *fgl2* protein was expressed in H5 cells. Chinese hamster ovary (CHO)-K1 cells were cultured in DMEM supplemented with 2 mM L-glutamine and 10% FBS. All tissue culture reagents were purchased from Life Technologies (Mississauga, Ontario, Canada).

Expression of fgl2 protein

Fgl2 protein with a tandem repeat of six histidine residues followed by an enterokinase cleavage site fused to the N terminus was prepared according to the Invitrogen Insect Expression System protocol. Briefly, a 1.4-kb cDNA encoding murine fgl2 was amplified using the forward primer 5′-TGCCGCACTGGATCCATGAGGCTTCCTGGT-3′ (with the methionine start codon underlined) and the reverse primer 5′-TTATGGCTTGAAAT TCTTGGGC-3′ (nt 1283–1302 relative to the ATG start codon). Amplification was performed for 25 cycles for 2 min at 96°C, 2 min at 55°C, and 3 min at 72°C. The PCR product was cloned into the *Eco*RI and *Bam*HI sites of the vector pBlueBacHis2A (Invitrogen).

Recombinant baculoviruses were generated by cotransfecting Sf9 cells with 2 μg pBlueBacHis2A carrying fgl2 insert plus 1 μg linearized *Autographa californica* multiple nuclear polyhedrosis virus DNA using the Bac-N-Blue transfection kit (Invitrogen). Putative recombinant viruses were screened for the presence of fgl2 by PCR followed by three rounds of viral plaque purification. The sequence of the recombinant baculovirus containing murine *fgl2* cDNA was confirmed by an automated DNA sequencer (model no. 377; PE Applied Biosystems, Foster City, CA).

His-tagged *fgl2* protein was purified using a nickel resin column according to Invitrogen's protocol. Briefly, monolayers of H5 cells were infected with the recombinant baculovirus at a multiplicity of infection of 5. Seventy-two hours later, the infected cells were harvested by centrifugation and lysed in 6 M guanidinium hydrochloride, 20 mM sodium phosphate, and 500 mM NaCl. The soluble material was mixed with 50% slurry of ProBond nickel-nitrilotriacetic acid (Ni-NTA) resin (Invitrogen) for 1 h at 4°C. After washings, bound *fgl2* protein was eluted with 8 M urea and 20 mM sodium phosphate (pH 5.3) with 150 mM NaCl. The pH of the eluted protein was adjusted to 7.2 immediately upon elution, and the protein was renatured by dialyzing against urea-saline buffers (150 mM NaCl (pH 7.2)) with successive decreases in urea concentrations (6, 4, 2, and 1 M) and finally against TBS (10 mM Tris, 150 mM NaCl (pH 7.2)). The dialyzed material was concentrated and soluble *fgl2* protein was collected by spinning at 14,000 rpm for 10 min to remove insoluble particulates. Protein concentrations were determined by BCA assay (Pierce, Rockford, IL).

Homogeneity of purified soluble *fgl2* protein was evaluated by SDS-PAGE and confirmed by Western blot probed with anti-*fgl2* Ab as previously described (13). Proteins were stained directly using Coomassie brilliant blue or were transferred to nitrocellulose, and then were probed using polyclonal rabbit anti-mouse *fgl2* IgG as the primary Ab. The secondary Ab used for immunoblotting was affinity-purified donkey anti-rabbit IgG conjugated to HRP (Amersham, Little Chalfont, U.K.) and the blot was visualized with an immunochemiluminescent kit (Amersham).

Reconstitution of purified fgl2 protein into phospholipid vesicles

Purified soluble *fgl2* protein was incorporated into phospholipid vesicles based on the detergent dialysis technique previously described (20–22). Briefly, hen egg phosphatidyl-L-serine (PS) and bovine brain phosphatidylcholine (PC) (Sigma-Aldrich, St. Louis, MO) in chloroform were combined to give different ratios of PS:PC (ranging from 0:100 to 40:60) and dried to a thin layer on the walls of a borosilicate glass tube under a stream of dry nitrogen and evacuated for 2 h to remove the resident traces of solvent. The lipid film (16 mg of total lipid content) was resuspended in 3 ml of 20 mM HEPES, 150 mM NaCl, and 80 mM octyl glucoside (pH 7; Sigma-Aldrich), and 20 μM of the lipid mixture was mixed with various concentrations of purified *fgl2* protein. The mixed micellar solution was placed in microdialysis chambers (Pierce) and dialyzed against successive changes of HEPES-saline buffer (20 mM HEPES, 0.15 M NaCl (pH 7.2)) for 96 h at room temperature. Following dialysis, samples were centrifuged at 14,000 rpm for 30 min to remove particulates. As a negative control, BSA was also reconstituted into the phospholipid membranes. All the preparations were stored at 4°C and 100 μl of each sample was used to assay for the coagulation activity.

Expression of truncated and mutated fgl2 proteins in CHO cells

The 1.4-kb cDNA encoding the murine fgl2 was amplified as previously described and the PCR product was cloned into the mammalian expression vector pCR3.1-Uni with the TA Cloning kit (Invitrogen) for the expression of wild-type *fgl2* protein. Using primer combinations in Table I, various truncated fgl2 constructs were generated. All truncated PCR products were amplified with the same 5′ primer but various 3′ primers with a stop codon (underlined in Table I) incorporated in the primer sequence and cloned into the vector pCR3.1-Uni with the TA Cloning kit. Sequences of the truncated constructs were confirmed by an automated DNA sequencer.

As we had postulated that *fgl2* had serine protease activity, serine residues 89, 136, and 426 were individually mutated to alanines to evaluate the effect on *fgl2* coagulation activity. Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol with primer containing the appropriate base substitutions as shown in Table I, such that serine residues were substituted individually by alanines. All sequences of the mutated constructs were confirmed by an automated DNA sequencer.

CHO-K1 cells were transfected with various plasmid DNA *fgl2* constructs using the Lipofectamine Plus Reagent kit (Life Technologies). Three days after transfection, the transfected cells were trypsinized and split onto new plates. After incubation for 24 h to allow the cells attach onto the plate, a neomycin analog, G418 (800 μg/ml; Life Technologies), was added to the culture and incubated until the plate became ~70% confluent for 10–14 days. Colonies were then selected and cultured with G418 (400 μg/ml) to generate stable cell lines by changing medium every 3–4

Table I. *Sequences of primers used to generate* fgl2 *PCR products for preparing the truncated and mutated fgl2 proteins*[a]

Constructs	Sequences
3′ Truncation constructs	
Forward primer	5′-TGC CGC ACT GCA AGG ATG AGG-3′ (−15 to 6)
Reverse primers	
Fgl12-aa1/386	5′-CTG GTG GTA TTA TTT GCC ATT-3′ (1149–1169)
Fgl2-aa1/336	5′-CT GAA ACG CTA GGC ATC CCC-3′ (1000–1019)
Fgl2-aa1/284	5′-CTT ACT CTA GGT CAG AAG ATG-3′ (846–866)
Fgl2-aa1/231	5′-CC CAT GGT CTA CAT GTC ACA-3′ (685–704)
Fgl2-aa1/181	5′-GCT GGG ACA CTA GGA ACA CTT-3′ (534–554)
Fgl2-aa1/135	5′-CTT CAG CTC TTA GGA CAG CTT G-3′ (396–407)
Serine-mutated constructs	
Ser^{425}Ala (sense primer)	5′-GGC TAC AAG GCC TCC TTC AAA-3′ (1266–1286)
Ser^{135}Ala (sense primer)	5′-AAG CTG TCC GCA GAG CTG AAG-3′ (396–416)
Ser^{89}Ala (sense primer)	5′-GCA GTG GAC GCT CTG AAG AAA-3′ (258–278)

[a] Truncated fgl2 constructs were generated using the same forward primer with an ATG start site and their corresponding reverse primer with a stop codon (TTA or CTA) as shown (underlined). Mutated PCR products were prepared by mutating the serine codons into alanines (underlined) using primers containing appropriate base substitutions. The nucleotide position of the primers relative to the downstream of the ATG start codon of *fgl2* cDNA are indicated in parentheses.

days. The cells were washed briefly with ice-cold PBS and spun at 2500 rpm for 5 min at 4°C. Cell pellets were then frozen immediately in liquid nitrogen and stored at −80°C until further analysis. Expression of *fgl2* proteins was verified by Western blotting and their prothrombinase activity was analyzed in a one-stage clotting assay.

One-stage clotting assay

Samples of purified soluble *fgl2* protein reconstituted into phospholipid vesicles were analyzed for their ability to accelerate the clotting time of recalcified normal human platelet-poor plasma in a one-stage clotting assay as previously described (23). Phospholipid vesicles alone or reconstituted with BSA were used as negative controls. In addition, stably transfected CHO-K1 cells (2×10^6 cells) subjected to three cycles of freeze/thawing and diluted in 100 μl of RPMI 1640 medium were assayed for their coagulation activity. In brief, samples (100 μl) were mixed with 100 μl of citrated normal human platelet-poor plasma and 100 μl of 15 mM $CaCl_2$ (prewarmed to 37°C), and the time required for the appearance of an insoluble fibrin clot with manual rocking at 37°C was recorded.

Additional one-stage clotting assays were performed with human plasmas deficient in coagulation factor II, VII, or X (Helena Laboratories, Beaumont, TX) to determine the factor dependence of relipidated *fgl2* prothrombinase activity. Data were expressed as the mean ± SE of triplicate assays.

Effects of protease inhibitors on fgl2 prothrombinase activity

Assessment of the susceptibility of *fgl2* prothrombinase to protease inhibitors was performed as previously described (23). Purified *fgl2* protein (150 nM) reconstituted into PCPS (75% PC:25% PS; 20 μM) was incubated with each of the following protease inhibitors: antithrombin III (10 μg/ml) in the presence of heparin (1 U/ml), soybean trypsin inhibitor (10 μg/ml), 4-aminobenzamidine (1 mM), aprotinin (10 U/ml), PMSF (1 mM), and diisopropylfluorophosphate (DFP; 2.5 and 10 mM; Sigma-Aldrich). Following a 15-min incubation with protease inhibitor at 22°C, 10 μl of ^{125}I-labeled prothrombin and 10 μl of 25 mM $CaCl_2$ were added, and the reactions were incubated for an additional 30 min at 37°C. To stop the reaction, 1% SDS and 1% EDTA were added and ^{125}I-labeled prothrombin cleavage was assessed by SDS-PAGE and soft laser scanning densitometry (Zeineh soft laser; Biomed Instruments, Chicago, IL). Human factor Xa, derived from factor X by cleavage with RVV, in the presence of phospholipids was treated identically as positive control. The data were normalized to the maximum amplitude of ^{125}I-labeled prothrombin cleavage when no inhibitor was used (defined as 0% inhibition) to facilitate comparison.

Measurement of rates of thrombin generation

Kinetics of thrombin generation by relipidated *fgl2* (150 nM plus 20 μM PL with 25% PS) was measured using a chromogenic substrate of thrombin, Spectrozyme TH (H-D-hexahydrotyrosyl-L-alanyl-L-arginine-*p*-nitroanalide-diacetate; American Diagnostica, Montreal, Canada). The initial rates of thrombin generation by the reconstituted *fgl2* protein with 7 μM prothrombin over a range of $CaCl_2$ concentrations (0–20 mM) were also quantitated. The effect of purified human factor Va (0.5–500 nM; Hematologic Technologies, Essex Junction, VT) on the murine *fgl2* prothrombinase activity at 0.7 μM prothrombin was also determined. Kinetics parameters of thrombin generation were obtained by measuring the initial rates of thrombin generation at various concentrations of prothrombin (0.01–250 μM) with a final concentration of 5 mM $CaCl_2$. The effects of factor Va (500 nM) on the K_m and V_{max} of relipidated *fgl2* was determined after incubating with factor Va for 1 min at 37°C and addition of prewarmed prothrombin in the assay buffer. The assay buffer used in all experiments, unless otherwise noted, was 5 mM $CaCl_2$, 10 mM HEPES, and 150 mM NaCl (pH 7.4). The initial rates of activation (<10% of substrate used) were determined. Reactions were monitored over time by quenching aliquots (10 μl) of the reaction in EDTA buffer (120 μl; 10 mM HEPES (pH 7), 100 mM NaCl, 5 mM EDTA, 1 mg/ml BSA, 1 mg/ml polyethylene glycol 8000) and measuring the rate of hydrolysis of Spectrozyme TH (20 μM), and the appearance of the chromophore, *p*-nitroanilide acetate, was monitored over time at 405 nm. The concentration of thrombin formed was calculated by comparison to a standard curve generated with purified human thrombin (Sigma-Aldrich). Pure prethrombin 1 and prethrombin 2 are known to have negligible amidase activity on Spectrozyme TH. Kinetic values were derived from the least-squares fit of the data based on the Michaelis-Menten equation (SigmaPlot). Rates of thrombin formation were calculated and expressed as moles of thrombin formed per minute per mole of *fgl2*. K_m and V_{max} values were determined by statistical analysis of the Lineweaver-Burk plots as described by Eisenthal and Cornish-Bowden (24) using the software SigmaPlot 2001 Enzymatic Kinetics (Sigma-Aldrich).

Flow cytometric analysis of fgl2 expressed on MHV-3 infected peritoneal macrophages

Female BALB/cJ mice, 6–8 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME) and were fed a standard chow diet and allowed to acclimatize for 1 wk before use. Peritoneal exudative macrophages from BALB/cJ mice were harvested in ice-cold PBS 4 days after i.p. injection of 1.5 ml of 5% thioglycolate (Difco, Detroit, MI) as previously described (25, 26). The cells were washed twice in cold PBS and resuspended in RPMI 1640, 2% FBS at 2×10^6 cells/ml. This procedure consistently yielded a >85% macrophage cell population confirmed by staining with anti-Mac1 (CD11b/CD18) Ab (Cedarlane Laboratories, Hornby, Ontario, Canada), with >98% viability by trypan blue exclusion. Cells were incubated for 1 h at 37°C before experimentation. Infection of peritoneal macrophages by MHV-3 was performed as previously described (25, 26). In brief, MHV-3 with a titer of 5×10^6 PFU/ml was used to infect the purified peritoneal macrophages at a multiplicity of infection of 5. After 8 h of infection, infection was stopped by placing the cells on ice.

Expression of *fgl2* on peritoneal macrophages was performed by flow cytometry. The MHV-3-infected macrophages were stained with biotinylated polyclonal rabbit anti-*fgl2* Ab at 4°C for 30 min in the dark. After two washes, cells were stained with streptavidin-PE (Cedarlane Laboratories) for 30 min at 4°C and finally analyzed using a COULTER Epics-XL flow cytometer (Beckman Coulter, Fullerton, CA).

Statistical analysis

The results were calculated as means ± SEM in all figures and tables; the means were compared using the analysis of variance by Student's *t* test using the software Statistix 7 (Analytical Software, Tallahassee, FL). A p value ≤ 0.05 was considered statistically significant.

Results

Expression of fgl2 protein in a baculovirus system

Murine *fgl2* protein was generated using the baculovirus expression system as described above. Fig. 1*a* shows a SDS-PAGE of *fgl2* baculovirus-infected H5 cells under reducing conditions followed by Western blot analysis. It can be seen that Coomassie blue staining (Fig. 1*b*) showed a dominant band at the same molecular size of ~65 kDa, comparable to the size of the *fgl2* protein previously estimated (13, 25).

Coagulation activity of fgl2 protein requires phospholipids and calcium

Initial studies using one-stage clotting analysis showed that the *fgl2* protein expressed by lysates of recombinant baculovirus-infected H5 cells exhibited coagulation activity with a dependence on $CaCl_2$. In contrast, uninfected H5 cells and cells infected with the wild-type baculovirus were devoid of coagulation activity (Table II). To characterize the molecular basis of *fgl2* prothrombinase activity in detail, we first purified *fgl2* protein. Purified *fgl2* protein did not exhibit any measurable coagulation activity consistent with reports indicating a phospholipid backbone is required for most clotting enzymes to manifest their activity (27–29) (Table II). Table II shows that purified *fgl2* protein reconstituted into phospholipid vesicles containing 10 or 25% PS had coagulation activity. Similar to data on the factor Xa/Va complex, phospholipid vesicles consisting of 25% PS also maximized recovery of prothrombinase activity, whereas no activity was detected when *fgl2* protein was incorporated into phospholipid vesicles devoid of PS. Unlike factor Xa (30), soluble purified *fgl2* protein exhibited coagulation activity only after incorporation into PS-containing vesicles, whereas no activity was detected when *fgl2* was mixed with PS containing vesicles without reconstitution (data not shown).

Using factor-deficient plasmas, we demonstrated that the coagulation activity of *fgl2* was dependent only on factor II (prothrombin) and thus behaved as a direct prothrombinase (Table III).

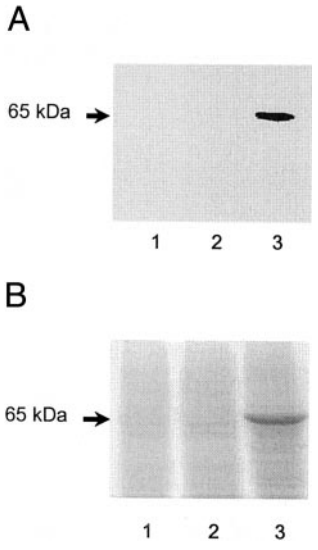

FIGURE 1. Expression of *fgl2* protein in a baculovirus system. *a*, Western blot analysis of *fgl2* expression in H5 cells infected with the recombinant baculovirus containing the *fgl2* insert. Homogenates of H5 cells infected with wild-type and recombinant baculoviruses were analyzed. Each lane was loaded with 20 μg of cell lysate and the blot was probed with polyclonal rabbit anti-mouse *fgl2* IgG as described in *Materials and Methods*. *Lane 1*, lysate of the uninfected H5 cells; *lane 2*, lysate of the wild-type baculovirus-infected H5 cells; *lane 3*, lysate of the recombinant baculovirus-infected H5 cells. *b*, SDS-PAGE followed by Coomassie blue staining of *fgl2* protein purified from recombinant baculovirus-infected H5 cells showed a dominant band consistent with the expression of *fgl2*. Cell lysates prepared under denaturing condition were incubated with Ni-NTA resin and proteins bound to Ni-NTA resin were eluted. All eluted proteins (10 μg) were analyzed on 10% SDS-PAGE and the gel was stained with Coomassie blue. *Lane 1*, eluted proteins from lysate of the uninfected H5 cells; *lane 2*, eluted proteins from lysate of the wild-type baculovirus-infected H5 cells; *lane 3*, eluted proteins from lysate of the recombinant baculovirus-infected H5 cells.

Kinetics of thrombin generation

Purified *fgl2* protein (150 nM) reconstituted into 20 μM PCPS (75% PC:25% PS) exhibited maximal coagulation activity and was used for kinetic analyses. As shown in Fig. 2, optimal *fgl2* prothrombinase activity of this preparation was reached with 5 mM $CaCl_2$. No prothrombinase activity was detected in the absence of $CaCl_2$.

Before performing kinetic studies, we confirmed that the rate of thrombin generation by the reconstituted *fgl2* protein was constant in time and proportional to the amount of *fgl2* present in the reaction mixtures. Using three concentrations of *fgl2* (15, 50, and 150 nM), the amount of thrombin generation was constant for up to 15 min and was proportional to the amount of *fgl2* reconstituted into the vesicles. No further increase in the rate of thrombin generation was observed when 300 nM *fgl2* reconstituted into PCPS vesicles was tested (data not shown). We then measured the catalytic function of *fgl2* by evaluating the initial rate of thrombin generation at different concentrations of substrate, prothrombin. The data presented in the form of Michaelis-Menten (Fig. 3*a*) and Lineweaver-Burk (Fig. 3*b*) plots show that relipidated *fgl2* behaves as an enzyme with a K_m value for prothrombin of 8.3 $\pm$ 1.1 μM and V_{max} value of 6 $\pm$ 0.2 mol of thrombin generated per minute per mole of *fgl2*.

Because factor Va is known to greatly enhance the catalytic activity of factor Xa, we next examined the potential role of factor Va on the catalytic efficiency of *fgl2*. The rate of formation of thrombin at different concentrations of factor Va ranging from 0.5 to 500 nM with 0.7 μM prothrombin substrate was monitored. Factor Va enhanced the catalytic efficiency of *fgl2* in a dose-dependent fashion, and the rate of thrombin generation was maximally reached when 500 nM of factor Va was used (Fig. 4*a*). The Lineweaver-Burk plot (Fig. 4*b*) suggested that factor Va (500 nM) enhances the catalytic efficiency of *fgl2* by increasing the V_{max} value to 3670 $\pm$ 87 mol of thrombin generated per minute per mole of *fgl2* and decreasing the K_m value to 7.2 $\pm$ 0.8 μM.

To further demonstrate that relipidated murine *fgl2* protein had prothrombinase activity, we performed a ^{125}I-labeled prothrombin cleavage assay as previously described (23). Fgl2 protein in the absence of phospholipid failed to cleave prothrombin. However, when *fgl2* was reconstituted into PCPS-containing vesicles, in the presence or absence of factor Va, characteristic prothrombin cleavage products were observed. In comparison to cleavage fragments generated by factor Xa, a unique band of ~24 kDa was seen when prothrombin was cleaved by *fgl2*, as we have previously reported (Ref. 17 and data not shown).

Table II. *Coagulation activity of* fgl2 *protein*

Samples	Clotting Time[a] (sec/2 $\times$ 10^6 cells)
Insect cell lysates[b]	
Uninfected H5 cells	256 $\pm$ 6
H5 cells infected with WT-*AcMNPV*	126 $\pm$ 2[c]
H5 cells infected with recombinant baculovirus	73 $\pm$ 3[c]
Purified *fgl2* protein	232 $\pm$ 6
Relipidated *fgl2*[d]	
Fgl2 protein alone	>240
Fgl2 + 100% PC	>240
Fgl2 + 90% PC:10% PS	150 $\pm$ 5[e]
Fgl2 + 75% PC:25% PS	110 $\pm$ 4[e]
Fgl2 + 60% PC:40% PS	>240

[a] Data (measured in seconds for every 2 $\times$ 10^6 cells) represent the mean $\pm$ SE of three experiments done in triplicate (n = 9).

[b] Lysate of recombinant baculovirus-infected H5 cells expressing *fgl2* protein shows significant coagulation activity compared to cells expressing no *fgl2* and to the purified *fgl2* protein. The uninfected, wild-type baculovirus (WT-*AcMNPV*)-infected, recombinant baculovirus-infected H5 cells (2 $\times$ 10^6 cells) and the purified *fgl2* protein were assayed for their ability to accelerate the clotting time of recalcified normal platelet-poor plasma.

[c] Significantly different from uninfected H5 cells, with $p < 0.01$.

[d] Purified *fgl2* protein did not exhibit prothrombinase activity, but the activity was restored when *fgl2* was reconstituted into membrane vesicles having appropriate PC to PS. Purified *fgl2* protein (150 nM) was reconstituted into 20 μM of phospholipid vesicles and the mixtures were assayed for their ability to accelerate the clotting time of recalcified normal platelet-poor plasma.

[e] Significant recovery of activity compared to purified *fgl2* protein alone, with $p <$ 0.001.

Table III. *One-stage clotting assay using plasmas deficient in various coagulation factors suggests that the coagulation activity of* fgl2 *was dependent on factor II but not factor VII or X*[a]

Plasma	Clotting Time (s)
Normal	110 $\pm$ 4
Deficient in:	
Factor VII	108 $\pm$ 6
Factor X	120 $\pm$ 4
Factor II	250 $\pm$ 3[b]

[a] Purified *fgl2* protein (150 nM) reconstituted into 20 μM of PCPS (75% PC:25% PS) was assayed for their ability to shorten the clotting time of human plasmas deficient in factors II, VII, or X as described in *Materials and Methods.* Results represent the mean $\pm$ SE of three experiments done in triplicate (n = 9).

[b] Significant loss of activity compared to result using normal plasma, with $p <$ 0.001.

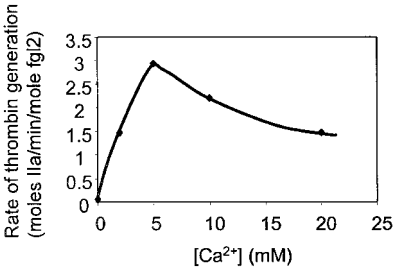

FIGURE 2. Effect of Ca^{2+} on the rate of thrombin generation by *fgl2* prothrombinase. Purified *fgl2* protein (150 nM) reconstituted into 20 μM 75% PC:25% PS membrane vesicles in 10 mM HEPES, 150 mM NaCl, and 7 μM prothrombin as substrate with various amounts of $CaCl_2$; at 0 mM-$CaCl_2$, 1 mM EDTA was present in the reaction mixture. Following initiation of the reaction at 37°C, aliquots were quenched and initial rates of thrombin generation were analyzed by measurement of the hydrolysis of Spectrozyme TH (20 μM) as described in *Materials and Methods*. The rate of thrombin formation at each calcium concentration was then calculated and expressed as moles of thrombin (IIa) per minute per mole of *fgl2* protein reconstituted into the phospholipid vesicles.

Identification of regions and residues required for fgl2 coagulation activity

To determine the regions of *fgl2* protein required for *fgl2* coagulation activity, six truncated proteins were stably expressed in CHO-K1 cells. The wild-type *fgl2* protein (*fgl2*-aa1/432) and the various truncated proteins were expressed at comparable levels as determined by Western blot analysis (data not shown). One-stage clotting assays were performed to determine whether the expressed proteins retained clotting activity. As shown in Fig. 5*a*, truncated proteins with deletion of amino acids 387–432 or 337–432 (i.e., *fgl2*-aa1/386 and *fgl2*-aa1/336, respectively) retained coagulation activity. Truncated proteins with amino acids 285–432, 232–432, 182–432, and 136–432 deleted (i.e., *fgl2*-aa1/284, Fgl2-aa1/231, Fgl2-aa1/181, and Fgl2-aa1/135, respectively) did not exhibit coagulation activity. Inactive proteins lacked residues 285–335, suggesting that one or more of these amino acids were critical for *fgl2* prothrombinase activity.

Although no classical Ser/His/Asp triad of most serine proteases was detected in the critical *fgl2* sequence, the presence of the SXXK motifs suggested that *fgl2* belonged to the clan SE of serine proteases (31). By analyzing the amino acid sequence, we identified three serine residues (Ser^{89}, Ser^{135}, and Ser^{425}) that might be critical if *fgl2* were a serine protease. To test the relevance of these

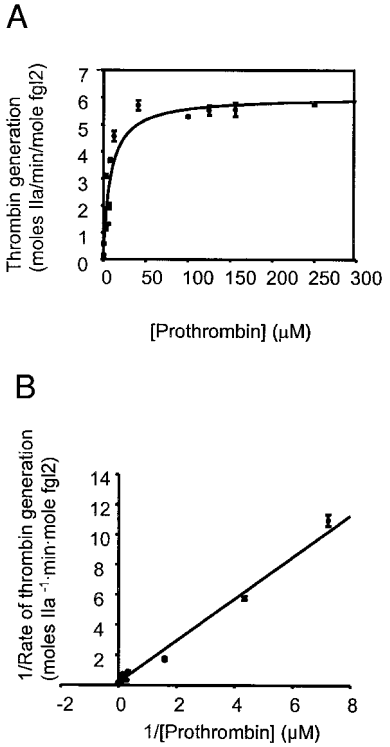

FIGURE 3. The Michaelis-Menten (*a*) and Lineweaver-Burk (*b*) plots of *fgl2* prothrombinase converting prothrombin to thrombin. Fgl2 protein (150 nM) reconstituted into 75% PC:25% PS (20 μM membrane vesicles was incubated with varying concentrations of prothrombin (0–250 μM)). Following initiation of the reaction at 37°C, aliquots were quenched and initial rates of thrombin generation were analyzed by measurement of the hydrolysis of Spectrozyme TH (20 μM) as described in *Materials and Methods*. The rate of thrombin formation at each prothrombin concentration was then calculated and expressed as moles of thrombin (IIa) per minute per mole of *fgl2* protein reconstituted into the phospholipid vesicles. The Lineweaver-Burk figure is plotted as reciprocals of rate vs prothrombin concentration.

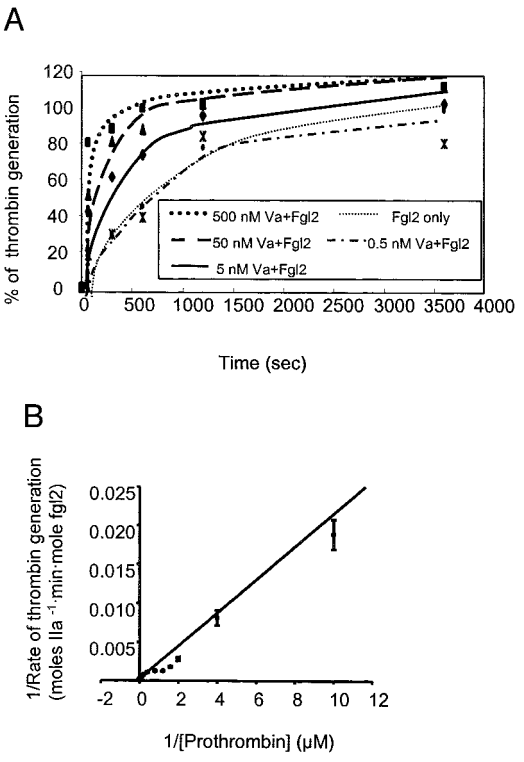

FIGURE 4. Effect of purified human factor Va on the rate of thrombin generation by *fgl2* prothrombinase. *a*, Rates of thrombin generation with different amounts of human factor Va. Initial rates of thrombin generation by purified *fgl2* protein (150 nM) reconstituted into 20 μM 75% PC:25% PS membrane vesicles with 0.7 μM prothrombin as substrate in 10 mM HEPES and 150 mM NaCl containing various amounts of human factor Va (0.5–500 nM) were analyzed. Following initiation of the reaction at 37°C, aliquots were quenched and initial rates of thrombin generation were analyzed by measurement of the hydrolysis of Spectrozyme TH (20 μM) as described in *Materials and Methods. b*, The Lineweaver-Burk plot of *fgl2* prothrombinase with human factor Va (500 nM) converting prothrombin to thrombin. Fgl2 protein (150 nM) reconstituted into 75% PC:25% PS (20 μM) membrane vesicles with factor Va was incubated with varying concentrations of prothrombin (0–250 μM). The rate of thrombin formation at each prothrombin concentration was then calculated and expressed as moles of thrombin (IIa) per minute per mole of *fgl2* protein reconstituted into the phospholipid vesicles. Data are plotted as reciprocals of rate vs prothrombin concentration.

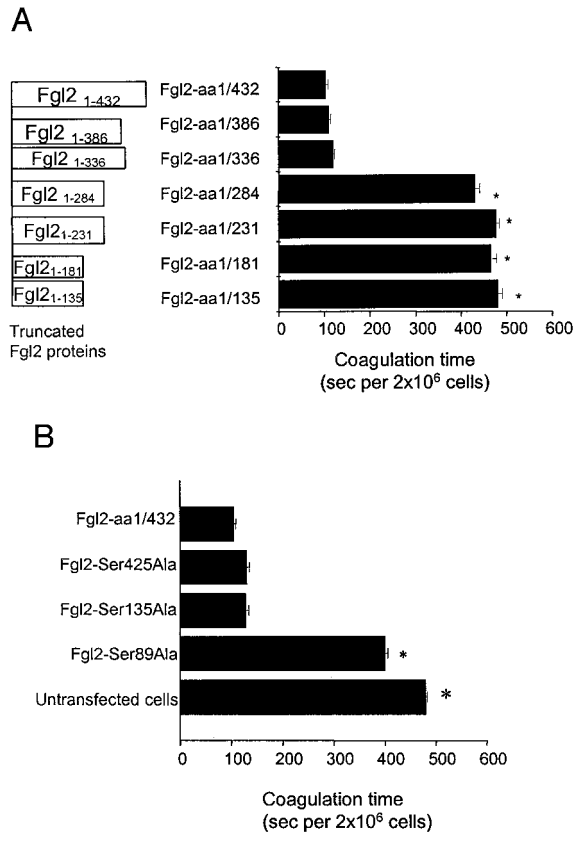

FIGURE 5. Region(s) and residue(s) critical for *fgl2* coagulation activity. *a*, Truncated *fgl2* proteins stably expressed in CHO-K1 cells exhibited different coagulation activities. *b*, Site-directed mutagenesis was performed to identify the critical residue(s) for the *fgl2* coagulation activity. One-stage clotting assay was performed to determine the ability of different truncated and serine-mutated *fgl2* proteins to accelerate the clotting time of recalcified normal platelet-poor human plasma as shown in *Materials and Methods*. Results were expressed as coagulation time (seconds per 2×10^6 cells). *, Significant loss of activity compared with full-length unmodified *fgl2* protein (Fgl2-aa1/432) with $p < 0.001$.

residues, we individually mutated them from serine into alanine. Wild-type and point-mutated murine *fgl2* constructs were stably expressed in CHO-K1 cells, and the cell lysates were analyzed for clotting activity; it was not feasible to prepare baculovirus clones and purify these mutants. The wild-type *fgl2* protein (Fgl2-aa1/432) and the various truncation mutants (i.e., Fgl2-Ser^{89}Ala, Fgl2-Ser^{135}Ala and Fgl2-Ser^{425}Ala) were expressed at comparable levels as detected by Western blot analysis (data not shown). Fig. 5*b* shows that mutating Ser^{135} or Ser^{425} to alanine did not alter the *fgl2* activity. However, the mutation Ser^{89}Ala completely abolished the activity, indicating that Ser^{89} residue was critical.

To compare *fgl2* prothrombinase activity to that of factor Xa, we examined the effect of protein inhibitors. As shown in Table IV, *fgl2* prothrombinase activity was not inhibited by antithrombin III in the presence of heparin, soybean trypsin inhibitor, 4-aminobenzamidine, aprotinin, or PMSF, whereas >80% inhibition of factor Xa activity was observed in the presence of these protease inhibitors. Furthermore, as little as 2.5 mM DFP completely inhibited *fgl2* prothrombinase activity, whereas 10 mM DFP was required for inhibition of factor Xa. The data indicated that *fgl2* protein directly cleaves prothrombin to thrombin in a manner that has the characteristic of a serine protease but differs from the serine protease activity of factor Xa.

Table IV. *Blockage of* fgl2 *prothrombinase activity by different protease inhibitors compared to their effects on factor Xa activity*[a]

Inhibitors	Inhibition (%)	
	Fgl2 prothrombinase	Factor Xa
None (buffer alone)	0	0
Antithrombin III	5 ± 3	90 ± 5[b]
Soybean trypsin inhibitor	5 ± 2	100 ± 0[b]
4-Aminobenzamidine	2 ± 1	90 ± 5[b]
Aprotinin	2 ± 2	95 ± 2[b]
PMSF	5 ± 3	100 ± 0[b]
DFP (2.5 mM)	95 ± 3[b]	10 ± 5
DFP (10 mM)	95 ± 2[b]	90 ± 3[b]

[a] Purified *fgl2* protein (150 nM) reconstituted into 20 μM of PCPS (75% PC:25% PS) vesicles were mixed with various protease inhibitors, and their ability to cleave prothrombin using ^{125}I-labeled prothrombin cleavage assay was examined. Data represent the mean ± SE of three experiments done in triplicate ($n = 9$) and are expressed as the percentage of inhibition for each inhibitor relative to control with no inhibitor.

[b] Significantly different from the sample that contained no inhibitor, with $p < 0.01$.

Expression of fgl2 on MHV-3-infected macrophages

MHV-3-infected macrophages of susceptible strain BALB/cJ mice express *fgl2* prothrombinase. Proteins involved in coagulation are either transmembrane proteins, such as tissue factor and thrombomodulin, or membrane-associated proteins, such as factors Xa, IXa, and VIIIa, that can be washed from the cell surface. To determine whether *fgl2* prothrombinase was transmembrane or membrane associated, we infected peritoneal macrophages of BALB/cJ mice with MHV-3 and tested for *fgl2* on the cell surface using flow cytometry analysis. The labeling procedure involves extensive washing. Fig. 6 shows a representative result. Mean fluorescence intensity of *fgl2* expression detected by specific anti-*fgl2* Ab increased from 0.8 ± 0.05 to 43.8 ± 3.8 after MHV-3 infection. Isotopic control Ab gave mean fluorescence intensity of 0.5 ± 0.08. We concluded that *fgl2* is a transmembrane protein, unlike factor Xa.

Discussion

The data presented in this paper demonstrate that murine *fgl2* protein exhibited coagulation activity dependent solely upon coagulation factor II (prothrombin), calcium, and a negatively charged phospholipid backbone. The kinetics of thrombin generation by relipidated *fgl2* prothrombinase was slow, similar to what has been previously reported for factor Xa alone (32). Addition of factor Va to Xa induces conformational changes of factor Xa and thereby increases its catalytic efficiency by a factor of 3000-fold (33). Interestingly, factor Va also enhanced the catalytic efficiency of *fgl2* in a dose-dependent fashion. Although the V_{max} value of thrombin generation for *fgl2* at 500 nM of factor Va was similar to that reported for factor Xa, the apparent K_m value was significantly higher, suggesting that murine *fgl2* has a lower affinity toward human prothrombin than human factor Xa. As the present studies were undertaken with molecules from different species, the calculated K_m value may underestimate the interaction of these molecules, reflecting species incompatibility. Previous data using coagulation proteins from different species support this contention (34). Further investigations are required to answer this question and also to determine how factor Va enhances *fgl2* activity.

Phospholipid is required for assembly of proteins of the coagulation cascade into functional form. For example, PS-containing membranes enhance the affinity of factor Xa for its cofactor Va by 75- to 800-fold (35). Also, PS is responsible for a 10-fold increase

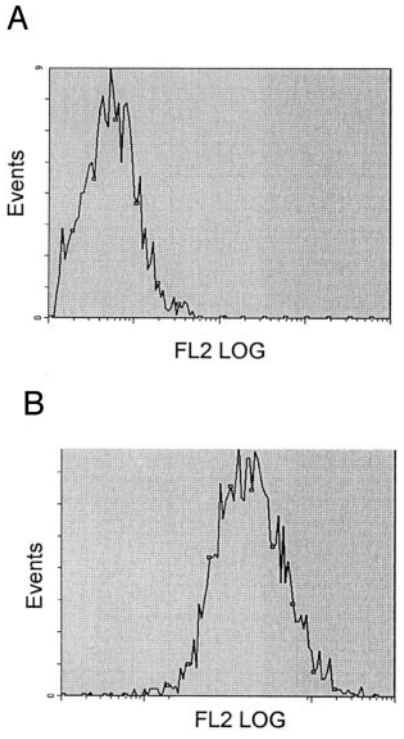

FIGURE 6. Flow cytometry analysis of MHV-3-infected macrophages suggests that *fgl2* is a transmembrane protein. Representative histograms of *fgl2* expression are shown. Shown are the uninfected (control) macrophages (*a*) and the MHV-3-infected macrophages (*b*) stained with biotinylated polyclonal anti-*fgl2* Ab then streptavidin-PE. The ordinate and the abscissa represent cell number and fluorescence intensity (FL2-H), respectively.

for factor IXa binding to factor VIIIa (29). In addition, PS-containing membranes increase the catalytic efficiency of the factors Va/Xa[30], factor VII/tissue factor (35), and the factors VIIIa/IXa complexes (29). Similarly, purified *fgl2* protein only exhibited prothrombinase activity when reconstituted into PS-containing vesicles. This supports our previous observation that murine *fgl2* coagulation activity was maximal after rearrangement of the negatively charged phospholipid (i.e., PS) from the inner leaflet of the cell membranes to the exterior leaflet during cycles of freeze-thawing of *fgl2*-expressing cells. In this paper, we report that a similar ratio of PS:PC is crucial for optimal murine *fgl2* coagulation activity. Whether relipidation of *fgl2* increases binding of *fgl2* to prothrombin or enhances catalysis of prothrombin by *fgl2* requires further study.

A series of truncated *fgl2* proteins were expressed in CHO-K1 cells to determine which region(s) were critical for its activity. Truncation of residues 336–432 did not affect *fgl2* prothrombinase activity, whereas deletion of amino acid residues of 285–335 completely abolished the activity. Truncations of murine *fgl2* upstream of amino acid residue 284 also resulted in an inactive protein. Deletion of such a large number of amino acid residues might have affected the proper conformation of *fgl2*. Alternatively, these deletions might have removed an exosite (prothrombin binding site) for the initial interaction of murine *fgl2* with prothrombin, distinct from the catalytically active site. A similar phenomenon has been reported in the recognition of prothrombin by the factor Xa/Va complex (36). A third mechanism for the loss of coagulation activity would be the removal of a domain required for a protein-phospholipid interaction. Fourth, the active site of *fgl2* could have been deleted. At the present time, we do not have evidence to support any of these possibilities, and additional experiments will have to be performed.

Studies by others have demonstrated that many coagulation proteins, including factors VII, IX, X, XII, and thrombin, have serine protease activity (8, 31, 37). It has been suggested that there are two main types of serine proteases: the trypsin-like and subtilisin-like, which have an identical arrangement of the "classical" catalytic Ser/His/Asp triad but in quite different protein scaffolds. Over 20 families (S1–S27) of serine proteases have now been identified and have been grouped into six clans (clans SA, SB, SC, SE, SF, and SG) on the basis of structural similarity and other functional features (31, 37). In this study, we demonstrated that murine *fgl2* protein has the characteristic of a serine protease based on the observation that the prothrombinase activity was inhibited by the serine protease inhibitor DFP. The concentration of DFP required for enzyme inhibition was well within the range that inhibits a number of known serine proteases that participate in the coagulation cascade (28). Moreover, the fact that murine *fgl2* prothrombinase activity was not inhibited by antithrombin III, soybean trypsin inhibitor, aprotinin, or 4-aminobenzamidine suggests that murine *fgl2* is a unique serine protease that behaves differently than other known coagulants.

Examination of the amino acid sequence of murine *fgl2* protein shows the presence of serine protease clan SE motifs SXXK with serine residues at positions 89, 135, and 425. Proteases such as *Escherichia* D-Ala-D-Ala peptidase A, *Streptomyces* R61 D-Ala-D-Ala peptidase, and *Actinomadura* R39 peptidase have been categorized into the clan SE family of serine proteases (31). No homology at the amino acids level besides the SXXK motif is found between *fgl2* protein and other members of the clan SE family. By site-directed mutagenesis, we have shown here that serine 89 is essential for murine *fgl2* prothrombinase activity. Results obtained using the truncated murine *fgl2* proteins are consistent with the site-directed mutagenesis results. Removal of residues 336–432 does not seem to be important for the expression of the clotting activity, consistent with the finding that mutation of serine 425 did not abolish the expression of coagulation activity. The mutation of serine 89 suggests the importance of the residue for the prothrombinase activity; however, as mentioned previously, the mechanism of the effect has not been determined. A similar case is observed for factor Xa when arginine 347 is mutated to asparagine, resulting in a selective reduction in the factor Va affinity of factor Xa (38).

Expression of murine *fgl2* protein with a coagulation activity on activated macrophages and endothelial cells has been reported. However, Marazzi et al. (19) have recently reported that murine *fgl2* protein is spontaneously secreted in vitro by freshly isolated human $CD4^+$ and $CD8^+$ T lymphocytes, but the secreted protein was devoid of coagulation activity. Thus, bioactive murine *fgl2* protein can express either as a transmembrane form or as a soluble form under different circumstances, but coagulation activity of murine *fgl2* requires phospholipid. The role of the soluble form of *fgl2* protein lacking coagulation activity remains undetermined.

In this paper we have defined a unique coagulant, *fgl2*. From the hemostasis perspective, murine *fgl2* bypasses both the intrinsic and extrinsic coagulation pathways to trigger prothrombin activation directly without the need of additional coagulation factors. However, similar to factor Xa, factor Va enhances the coagulation activity of murine *fgl2*. From the immunologic perspective, murine *fgl2* results in direct generation of thrombin, which plays a significant role in inflammation by amplifying the inflammatory response. For instance, thrombin causes endothelial cells to produce IL-8, which induces endothelial P-selectin expression and early neutrophil adhesion and activation (39, 40). Also, thrombin is a potent chemotaxin for monocytes and polymorphonuclear cells,

resulting in influx of massive infiltrates to the site of inflammation, which is the hallmark of diseases in which murine *fgl2* has been implicated in the pathogenesis (1, 2, 16). The characterization of *fgl2* and identification of serine 89 residue accounting for its prothrombinase activity will allow the development of novel strategies to prevent fulminant liver failure, cytokine-induced fetal loss, and other diseases in which *fgl2* plays a pathogenic role.

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