

# Basic residues of human group IIA phospholipase A<sub>2</sub> are important for binding to factor Xa and prothrombinase inhibition

## Comparison with other mammalian secreted phospholipases A<sub>2</sub>

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Human secreted group IIA phospholipase A<sub>2</sub> (hGIIA) was reported to inhibit prothrombinase activity because of binding to factor Xa. This study further shows that hGIIA and its catalytically inactive H48Q mutant prolong the lag time of thrombin generation in human platelet-rich plasma with similar efficiency, indicating that hGIIA exerts an anticoagulant effect independently of phospholipid hydrolysis under *ex vivo* conditions. Charge reversal of basic residues on the interfacial binding surface (IBS) of hGIIA leads to decreased ability to inhibit prothrombinase activity, which correlates with a reduced affinity for factor Xa, as determined by surface plasmon resonance. Mutation of other surface-exposed basic residues, hydrophobic residues on the IBS, and His48, does not affect the ability of hGIIA to inhibit prothrombinase activity and bind to factor Xa. Other basic, but not neutral or acidic, mammalian secreted phospholipases A<sub>2</sub> (sPLA<sub>2</sub>s) exert a phospholipid-independent inhibitory effect on prothrombinase activity, suggesting that these basic sPLA<sub>2</sub>s also bind to factor Xa. In conclusion, this study demonstrates that the anticoagulant effect of hGIIA is independent of phospholipid hydrolysis and is based on its interaction with factor Xa, leading to prothrombinase inhibition, even under *ex vivo* conditions. This study also shows that such an interaction involves basic residues located on the IBS of hGIIA, and suggests that other basic mammalian sPLA<sub>2</sub>s may also inhibit blood coagulation by a similar mechanism to that described for hGIIA.

**Keywords:** anticoagulant; blood coagulation; phospholipase A<sub>2</sub>; surface plasmon resonance; venom.

Secreted phospholipases A<sub>2</sub> (sPLA<sub>2</sub>s) are enzymes found in mammals and animal venoms that hydrolyse the fatty acid esterified at the *sn*-2 position of phospholipids [1–5]. They share common characteristics including low molecular mass (14–17 kDa), calcium-dependent catalytic activity, the presence of between five and eight disulfide bridges, and a number of conserved amino acids located in the calcium-binding loop and the catalytic site. In mammals, different sPLA<sub>2</sub>s have been reported and classified into groups IB, IIA, IIC, IID, IIE, IIF, V, and X [2–8]. Snake venom sPLA<sub>2</sub>s have been classified into groups IA, IB, IIA, IIB, and those from bee and lizard venoms belong to group III. The structural and enzymatic properties of human group IIA sPLA<sub>2</sub> (hGIIA) have been well documented, and this enzyme has been detected in various cells including macrophages, eosinophils and blood platelets [9]. hGIIA has

attracted particular attention with respect to inflammatory diseases because its level in body fluids correlates with the severity of pathological states [10,11]. hGIIA also appears to be involved in the killing of invading Gram-positive bacteria [12], exocytosis/degranulation processes [13,14], and the production of eicosanoids by stimulated inflammatory cells [15,16]. These various biological effects have been reported to be mainly related to the catalytic activity of hGIIA.

In platelets, hGIIA is associated with  $\alpha$ -granules and is released into the extracellular medium on activation by thrombin and collagen [17–19]. Once secreted, hGIIA does not participate in the production of eicosanoids and does not interfere with platelet stimulation [20,21]. On the other hand, hGIIA has been reported to exert an anticoagulant effect and may be involved in negative feedback during the initiation of thrombosis [22–24]. Platelet activation plays a central role in haemostasis and thrombosis, leading to the formation of the primary plug and to the appearance of anionic phospholipids at the membrane surface, which increase the efficiency of the coagulation process [25–27]. Platelets are also a rich source of factor V [28], which after activation to factor Va, participates in the formation of the prothrombinase complex [25,29]. In contrast with most other biological effects of hGIIA, it has been demonstrated that its anticoagulant effect is phospholipid-independent, and therefore has been proposed to be unrelated to its catalytic activity [23]. Indeed, hGIIA specifically inhibits the prothrombinase complex composed of factor Xa, factor Va, phospholipid and calcium, and this inhibitory effect is still

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**Abbreviations:** sPLA<sub>2</sub>, secreted phospholipase A<sub>2</sub>; hGIB, human group IB sPLA<sub>2</sub>; hGIIA, human group IIA sPLA<sub>2</sub>; hGV, human group V sPLA<sub>2</sub>; hGX, human group X sPLA<sub>2</sub>; IBS, interfacial binding surface; IC<sub>50</sub>, concentration of inhibitor producing 50% inhibition; mGIIA, mouse group IIA sPLA<sub>2</sub>; mGIID, mouse group IID sPLA<sub>2</sub>; PRP, platelet-rich plasma; rGIIA, rat group IIA sPLA<sub>2</sub>; rGV, rat group V sPLA<sub>2</sub>; RU, resonance unit; SPR, surface plasmon resonance.

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observed in the absence of phospholipids [23,24]. hGIIA forms a 1 : 1 complex with human factor Xa and prevents formation of the factor Xa-factor Va complex [30]. In addition, high concentrations of factor Va in the prothrombinase assay reversed the inhibitory effect of hGIIA, consistent with the view that hGIIA competes with factor Va for binding to factor Xa [30]. The relevance of this model, explaining the phospholipid-independent anticoagulant effect of hGIIA, remains, however, to be examined under more physiological conditions. Furthermore, the structural elements of hGIIA involved in the interaction with human factor Xa have not been identified. Several basic venom sPLA<sub>2</sub>s have been shown to exert anticoagulant activity, suggesting that basic residues are involved [31–34]. hGIIA is also a basic protein containing 13 lysine and 10 arginine residues which are scattered over the whole surface and form cationic clusters. Several of them lie on the surface of the molecule that contacts the lipid membrane, the so-called ‘interfacial binding surface’ (IBS) [35].

In this study, we first investigated whether the phospholipid-independent anticoagulant effect of hGIIA, based on the direct interaction of hGIIA with factor Xa, is also observed under more physiological conditions. We next examined by site-directed mutagenesis the residues of hGIIA involved in its interaction with factor Xa, with particular emphasis on basic residues. Finally, we tested other recently cloned mammalian sPLA<sub>2</sub>s for antiprothrombinase activity.

## EXPERIMENTAL PROCEDURES

### Materials

Human prothrombin, factor V and factor Xa were from Enzyme Research Laboratories (South Bend, IN, USA). Factor Va and gla-minus factor Xa were from Haematologic Technologies Inc. (Essex Junction, VT, USA). The chromogenic substrate for thrombin amidolytic activity, S-2238, was from Chromogenix (Cincinnati, OH, USA). BSA (fraction V), phosphatidylcholine (P-7763) and phosphatidylserine (P-6641) were from Sigma (St Louis, MO, USA). sPLA<sub>2</sub> purified from *Apis mellifera* venom was from Sigma. Mouse group IID (mGIID) and group IIA (mGIIA), and human group V (hGV) and group X (hGX) sPLA<sub>2</sub>s were expressed, refolded and purified as described previously [6,36]. The basic sPLA<sub>2</sub> CM-III from *Naja mossambica mossambica* venom and Ba IV sPLA<sub>2</sub> from *Bothrops asper* venom were purified as described [2,37]. Human group IB sPLA<sub>2</sub> (hGIB) was a gift from the late H. Verheij, rat group IIA (rGIIA) and group V (rGV) sPLA<sub>2</sub>s were from M. Janssen (Department of Membranes, Utrecht University, the Netherlands), and AmtxA was from I. Krizaj (Jozef Stefan Institute, Ljubljana, Slovenia).

### Preparation of wild-type and mutant hGIIA recombinant proteins

The expression vector pT7-7 (Pharmacia) was transfected into BL21(DE3) *Escherichia coli* strain [38]. The BamHI–HindIII fragment encoding hGIIA [39] was cloned into the expression vector to obtain a wild-type primary sequence for hGIIA (see Fig. 3). hGIIA was expressed as a fusion protein with a 12-amino-acid N-terminal extension ending in an arginine residue for tryptic liberation of the hGIIA with its native N-terminus, as previously described [23,39]. Wild-type (or mutant) active hGIIA protein was obtained from the refolded fusion protein by tryptic cleavage [23]. hGIIA and its mutants were finally purified sequentially on two SP-Sephadex columns,

tested for activity using a fluorescent substrate, and checked for purity by FPLC and SDS/PAGE as previously reported [20].

### Site-directed mutagenesis of hGIIA

DNA fragments coding for hGIIA mutants H48Q, K53G/R54E, K57N/R58L, A18S/A19G/L20S, and F24S were prepared by PCR using *Pwo* polymerase (Roche Biochemicals, Meylan, France). Two PCRs were performed with the wild-type hGIIA pT7-7 plasmid, using the NT2 primer (5′-GTAACTTC-CACCGTATGATCAAA-3′) and the antisense mutagenic oligonucleotide for the PCR<sub>1</sub> fragment; and the CT2 (5′-AAGCATTGGTAACTGTCAAGACC-3′) primer and the sense mutagenic oligonucleotide for the PCR<sub>2</sub> fragment. PCR<sub>1</sub> and PCR<sub>2</sub> fragments were mixed and a third amplification was performed in the presence of NT2 and CT2 primers to obtain the PCR<sub>3</sub> fragment which was then cloned into the pGEM-T cloning vector (Promega). Finally, the BamHI–HindIII fragment containing the mutated sequence was released from the pGEM-T vector and ligated into the pT7-7 vector. The L2S/V3G mutant was prepared by a single-step PCR with the CT2 primer and the L2S/V3G sense mutagenic oligonucleotide. All mutants were sequenced completely before expression by the Sanger method (Sequenase 2.0). Nucleotide sequences of sense mutagenic oligonucleotides were:

H48Q, 5′-GCGTTACCCAgGACTGCTGCT-3′;

K53G/R54E, 5′-GCTGCTACggtgaaCTGGAAAAACGT-3′;

K57N/R58L, 5′-CTGGAAAAcCtgGGTTGCGGTACC-3′;

A18S/A19G/L20S, 5′-CCGGTAAAGAAAtCcGgTtcTCCTAC-GGTTTCTACGG-3′;

F24S, 5′-GCTCTGTCCTACGGTTcCTACGGTTGCCACTG-CGGTGTGGTGGTTCGTGG-3′;

L2S/V3G, 5′-CCGGGGATCCATCGAAGGTCGGAACtccGg-TAACTTCCACC-3′.

Small letters corresponds to the mutated region of the hGIIA sequence. The other mutants, K74E, K87E, R92E, K74E/K87E/R92E, R7E/K10E/K16E, K100E, R108E, R132E, K53E/R54E/R58E, K53E/R54E/K57E/R58E, K38E/K110E/K115E/K116E, K38E/K124E/R127D, K124E/R127D, R7E/K10E/K16E/K124E/R127D, V3W were prepared as described previously [35,40].

### sPLA<sub>2</sub> assay

sPLA<sub>2</sub> activity was assayed with a fluorescent phospholipid analog, 1-palmitoyl-2-(10-pyrenyl)decanoyl-*sn*-glycero-3-phosphomethanol (Molecular Probes) used as a substrate as described previously [35]. Briefly, the reaction medium was prepared by sequential addition of: (a) 980 μL 50 mM Tris/HCl, pH 8, 50 mM KCl and 1 mM CaCl<sub>2</sub>; (b) 1.5 μM or 20 μM (final concentration) fluorescent substrate; and (c) 20 μL 10% fatty-acid-free BSA (Sigma) solution in water. The enzyme reaction was then initiated by adding 2–10 μL of the sample.

### Measurement of thrombin generation in human platelet-rich plasma (PRP)

Blood was obtained from five distinct healthy donors. The blood of each donor was separately used to investigate the effect of wild-type hGIIA (three experiments) and the catalytically inactive H48Q mutant of hGIIA (two experiments). PRP was prepared by centrifugation of fresh citrated blood (nine parts of blood to one part of 0.13 M trisodium citrate) at 250 g at 15 °C for 10 min. The platelet concentration was adjusted to 300 000 cells·μL<sup>-1</sup>. Thrombin generation in PRP

was measured as previously described [41,42]. In brief, 480  $\mu\text{L}$  PRP was incubated with 120  $\mu\text{L}$  buffer A (20 mM Hepes, 150 mM NaCl, 0.5  $\text{g}\cdot\text{L}^{-1}$  BSA, pH 7.35) for 4 min, and coagulation was initiated by adding 120  $\mu\text{L}$  0.1 M  $\text{CaCl}_2$ . The effect of hGIIA or the enzymatically inactive H48Q hGIIA was tested by adding these proteins to the reaction mixture 4 min later. Samples (10  $\mu\text{L}$ ) of the reaction mixture were taken at 1 min intervals and the level of free thrombin was calculated from amidolytic activity using a previously described computer program that takes into account the contribution of free thrombin and  $\alpha_2$ -macroglobulin-bound thrombin [41,42]. The thrombin-generation curve, the thrombogram, gives three important variables: (a) the lag time of thrombin formation, which is defined as the time from addition of the triggering solution to the time at which the thrombin concentration increases above 10 nM; (b) the height of the peak that corresponds to the maximal thrombin concentration that can be reached; (c) the endogenous thrombin potential, which is defined as the area under the thrombin-generation curve and is directly proportional to the amount of thrombin generated during the coagulation process.

### Coagulation tests with human blood plasma

Human control plasma (Diagnostica Stago, Asnieres, France) was reconstituted with 1 mL distilled water before use and diluted tenfold with saline buffer (0.15 M NaCl). The recalcification time of plasma was measured in the absence or presence of wild-type (or mutant) hGIIA as reported [23]. For each measurement, 300  $\mu\text{L}$  diluted human plasma was incubated for 3 min at 37 °C under gentle stirring, then the coagulation was started by the addition of 6 mM  $\text{CaCl}_2$ . The time required for clot formation in the presence of wild-type (or mutant) hGIIA was recorded and expressed as a percentage of the clotting time measured in the absence of hGIIA. Three independent experiments were performed.

### Measurement of prothrombinase activity

Phospholipid vesicles (phosphatidylcholine/phosphatidylserine, 9 : 1, w/w) were prepared as described by de Kruijff *et al.* [43]. Purified prothrombinase components were mixed and subsequently assayed at 37 °C in Tris-buffered saline (0.1 M Tris/HCl, 0.05 M NaCl, 0.5% BSA, 5 mM  $\text{CaCl}_2$ , pH 7.4) in the two following combinations: (a) factor V, factor Xa and phospholipid: 20  $\mu\text{M}$  factor Xa was incubated with 120  $\mu\text{M}$  factor V for 4 min, then the reaction was started with 5  $\mu\text{M}$  phospholipid and 200 nM prothrombin; (b) factor Va and factor Xa: 1 nM factor Xa was incubated with 1 nM factor Va for 4 min, then the reaction was started with 1  $\mu\text{M}$  prothrombin.

Activated prothrombin activity was determined at various time points as described [30]. Wild-type hGIIA (or mutant proteins) was added at the beginning of the 4 min preincubation period. The  $\text{IC}_{50}$  value, which corresponds to 50% inhibition of thrombin generation, was then determined for wild-type and mutant hGIIA, as well as for other sPLA<sub>2</sub>s, as reported [30].

### Surface plasmon resonance (SPR) experiments

Studies were performed using a BIAcore® 2000 system (BIAcore AB, Uppsala, Sweden). Immobilization of factor Xa (or gla-minus factor Xa) on the Sensor Chips CM5 surface was performed as reported [30]. The SPR signals for immobilized factor Xa on three different flow cells were found to be: 7400 resonance units (RU), 4000 RU and 2000 RU, where 1 RU corresponds to an immobilized protein concentration of

$\approx 1 \text{ pg}\cdot\text{mm}^{-2}$ . Unreacted moieties on the surface were blocked by ethanolamine. One independent flow cell of the same sensor chip, used as a control flow cell, was subjected to a 'blank immobilization'. All experiments were carried out at 25 °C in 10 mM Hepes, pH 7.4, 0.005% surfactant P20 and 150 mM NaCl. Different concentrations of hGIIA or its mutants (0.5, 1, 2, 4, 8, and 16  $\mu\text{g}\cdot\text{mL}^{-1}$ ) were injected in the same buffer in the presence of  $\text{CaCl}_2$  at 5 mM, at a flow rate of 10  $\mu\text{L}\cdot\text{min}^{-1}$ . Between each injection, surfaces were regenerated with 5  $\mu\text{L}$  1 M NaCl. Kinetic constants,  $k_{\text{on}}$  (association rate constant) and  $k_{\text{off}}$  (dissociation rate constant), were calculated using BIAcore BIAEVALUATION 3 software and a two-component model of interaction [44], and values were determined after subtraction of control signals with the control flow cell. To investigate the effect of salt concentrations on the factor Xa–hGIIA interaction, similar experiments were performed with higher NaCl concentrations (300 mM and 500 mM).

## RESULTS

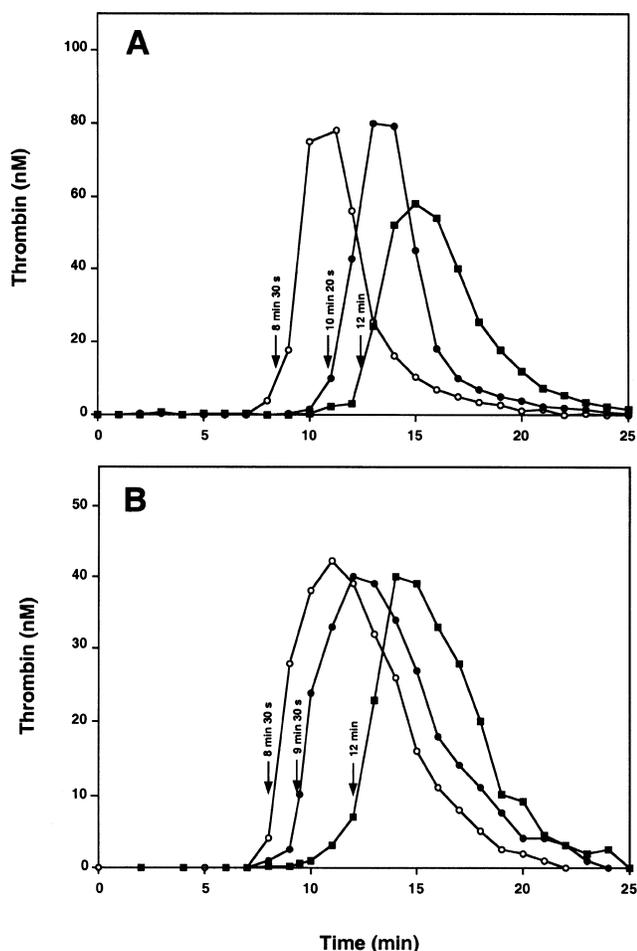
### Inhibitory effect of wild-type and catalytically inactive hGIIA on thrombin generation measured in human PRP

Previous experiments have shown that hGIIA inhibits the prothrombinase complex activity measured *in vitro* with a coagulation assay performed in the absence of platelets [30]. As platelets greatly increase the efficiency of blood coagulation and may counteract the anticoagulant effect of hGIIA, we analyzed whether hGIIA can still inhibit thrombin generation when measured under more physiological conditions with human PRP. We observed a large delaying effect of hGIIA on thrombin generation measured in PRP which depended on the concentration of hGIIA (Fig. 1A). The mean increases in lag time were 115% for 1  $\mu\text{M}$ , 140% for 2  $\mu\text{M}$  and 170% for 5  $\mu\text{M}$  hGIIA. The effect of hGIIA was saturable, as concentrations higher than 5  $\mu\text{M}$  did not lead to a further increase in the lag time of thrombin formation (data not shown). In a few cases, this delay was also associated with a moderate (15–30%) decrease in the endogenous thrombin potential, corresponding to the quantity of thrombin generated.

As natural phospholipids from plasma and activated platelets are present at physiological concentrations in the thrombin-generation assay with human PRP, the anticoagulant effect of hGIIA may be due to its enzymatic activity under such conditions. To investigate this possibility, we generated a catalytically inactive mutant of hGIIA by replacing His48 of the catalytic site with a glutamine (H48Q). The enzymatic activity of the H48Q mutant is less than 0.05% of that of the wild-type enzyme. As shown in Fig. 1B, the H48Q mutant shows the same efficiency in delaying thrombin generation as wild-type hGIIA (Fig. 1A). The average increases in the lag time were 115% for 0.5  $\mu\text{M}$  and 140% for 1.5  $\mu\text{M}$  H48Q hGIIA. This clearly demonstrates that hGIIA exerts an anticoagulant effect under *ex vivo* conditions and that this inhibitory effect is independent of its catalytic activity even when the source of phospholipid reflects physiological conditions.

### Wild-type and catalytically inactive hGIIA inhibit prothrombinase activity and bind to factor Xa with similar affinity

The prothrombinase complex was reconstituted *in vitro* in the presence or absence of phospholipids to determine whether hGIIA acts on phospholipids or protein coagulation factor(s).



**Fig. 1.** Effect of wild-type and catalytically inactive H48Q hGIIA on thrombin generation measured in human PRP. Thrombin generation (nM) was measured in human PRP as a function of time (min) as described in Experimental procedures. (A) Wild-type hGIIA (●, 1 μM; ■, 2 μM) or buffer (○, control), or (B) catalytically inactive H48Q hGIIA (●, 0.5 μM; ■, 1.5 μM) or buffer (○, control) were added to the reaction mixture 4 min after the addition of the triggering agent, CaCl<sub>2</sub>. The arrows indicate the lag time (min). Representative curves from five experiments are shown.

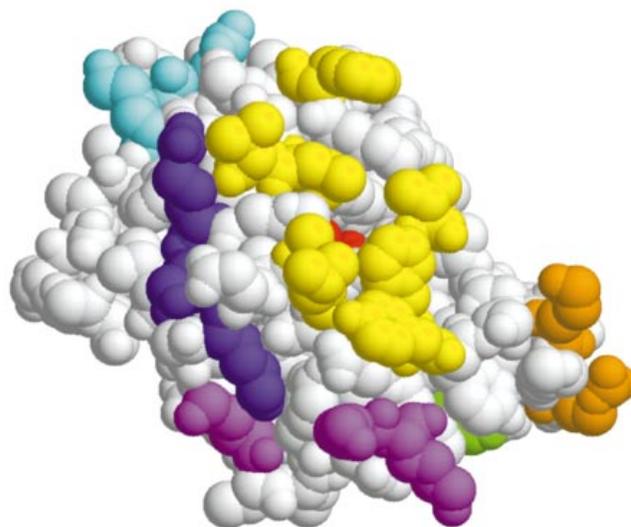
When the prothrombinase complex is composed of calcium, phospholipids, factor Xa, factor Va and prothrombin, it reflects the *in vivo* composition, but this assay does not allow discrimination between phospholipid-dependent and protein-dependent mechanisms. When the prothrombinase complex is reconstituted in the absence of phospholipids, any inhibition is due to a direct effect on a protein component. Table 1 shows that hGIIA inhibits prothrombinase activity with similar efficiency in the presence ( $IC_{50} = 80$  nM) and absence ( $IC_{50} = 50$  nM) of phospholipids. SPR data (Table 1) indicate that the inhibition of prothrombinase activity (measured in the absence of phospholipids) by hGIIA correlates with its binding to factor Xa with a  $k_{on}$  of  $310\,000\text{ M}^{-1}\cdot\text{s}^{-1}$ , a  $k_{off}$  of  $0.00155\text{ s}^{-1}$  and a  $K_d^{app}$  of 5 nM. We observed that the binding of hGIIA to factor Xa was completely abolished when higher salt concentrations (300 mM and 500 mM instead of 150 mM) were used during BIAcore experiments (Table 1). The  $IC_{50}$  values for H48Q hGIIA determined in the presence and absence of phospholipid were similar to those measured for wild-type hGIIA, and no changes in  $k_{on}$ ,  $k_{off}$  and  $K_d^{app}$  values were observed (Table 1), indicating that the inhibitory effect of

hGIIA measured *in vitro* is independent of its catalytic activity, but due to specific binding to factor Xa.

The gla domain of factor Xa has been shown to promote calcium-dependent binding of factor Xa to phospholipid [45]. As hGIIA inhibits prothrombinase activity both in the presence and absence of phospholipids, the interaction of hGIIA with factor Xa should be independent of the gla domain of factor Xa. This was investigated by replacing native factor Xa with factor Xa lacking the gla domain (gla-minus factor Xa) in the prothrombinase complex. As gla-minus factor Xa does not bind to phospholipid, prothrombinase activity was measured in the absence of phospholipid. We observed that the inhibitory effect of hGIIA on prothrombinase activity measured with gla-minus factor Xa ( $IC_{50} = 50$  nM) is similar to that measured with native factor Xa (Table 1). Moreover, the kinetic constants measured by SPR for the interaction between hGIIA and gla-minus factor Xa were similar to those determined with native factor Xa (Table 1). This indicates that the interaction of hGIIA with factor Xa does not involve the gla domain and that prothrombinase inhibition by hGIIA does not require the interaction of either factor Xa or hGIIA with phospholipids.

#### Involvement of basic residues of hGIIA in the binding to factor Xa

A number of exposed basic residues, i.e. R7, K10, K16, K38, K74, K87, R92, K110, K115, K116, K124 and R127, are located on or near the IBS of hGIIA. These residues form several basic clusters: R7/K10/K16; K110/K115/K116; K74/K87/R92 and K124/R127 (Fig. 2). Other exposed basic residues of hGIIA, i.e. K53, R54, K57, R58, and R132, lie on the face of the molecule that is opposite to the IBS, whereas residues K100 and R108 are located in another region. Various mutants of hGIIA were produced by replacing one or several of these basic residues with acidic ones and assayed for their ability to inhibit prothrombinase activity and to bind to factor Xa (Fig. 3 and Table 1). As the mutated side chains



**Fig. 2.** A view of the IBS of hGIIA. H48 of the catalytic site of hGIIA is indicated in red. Basic clusters of the IBS of the hGIIA are indicated in: purple for R7/K10/K16; mauve for K110/K115/K116; orange for K124/R127; cyan for K74/K87/R92; green for K38. Hydrophobic residues of the IBS of the hGIIA are indicated in yellow: L2, V3, A19, L20, F24, V31, F70 and Y119. The numbering system is that of Renetseder *et al.* [46].

**Table 1.** Effect on prothrombinase activity and FXa-binding kinetic parameters of hGIIA and mutants. PL, phospholipid.

	Inhibition of prothrombinase activity (IC <sub>50</sub> , nM)		Factor Xa-binding kinetic parameters			Specific activity % of wild-type
	Absence of PL	Presence of PL	k <sub>on</sub> (M <sup>-1</sup> ·s <sup>-1</sup> )	k <sub>off</sub> (s <sup>-1</sup> )	K <sub>d</sub> <sup>app</sup> = k <sub>off</sub> /k <sub>on</sub> (nM)	
Wild-type	50 ± 6	80 ± 10	3.1 ± 0.7 × 10 <sup>5</sup>	1.55 ± 0.4 × 10 <sup>-3</sup>	5	100
Wild-type <sup>a</sup>	52 ± 6	–	4.1 ± 0.2 × 10 <sup>5</sup>	1.60 ± 0.3 × 10 <sup>-3</sup>	3.9	
Wild-type <sup>b</sup>	–	–	0	0	–	
H48Q	35 ± 4	110 ± 5	3.5 ± 0.5 × 10 <sup>5</sup>	2.35 ± 0.4 × 10 <sup>-3</sup>	6.7	0.05
K53G/R54E	40 ± 4	65 ± 8				120
K57N/R58L	140 ± 10	295 ± 50	3.9 ± 0.2 × 10 <sup>5</sup>	1.35 ± 0.1 × 10 <sup>-3</sup>	3.5	115
K53E/R54E/R58E	90 ± 10	240 ± 50				102
K53E/R54E/K57E/R58E	110 ± 10	360 ± 50	2.5 ± 0.3 × 10 <sup>5</sup>	2.90 ± 0.5 × 10 <sup>-3</sup>	12	90
K100E	65 ± 10	95 ± 20				120
R108E	105 ± 13	150 ± 20				104
R132E	55 ± 6	145 ± 3				102
K74E/K87E/R92E	195 ± 20	290 ± 20	2.6 ± 0.4 × 10 <sup>5</sup>	15 ± 1 × 10 <sup>-3</sup>	58	120
K74E	85 ± 10	115 ± 3				97
K87E	155 ± 30	180 ± 5				110
R92E	60 ± 4	100 ± 5				103
K124E/R127D	180 ± 20	420 ± 30	2.3 ± 0.3 × 10 <sup>5</sup>	13.5 ± 2 × 10 <sup>-3</sup>	59	106
R7E/K10E/K16E	215 ± 25	310 ± 6	2.7 ± 0.3 × 10 <sup>5</sup>	14.5 ± 1 × 10 <sup>-3</sup>	54	108
R7E/K10E/K16E/K124E/R127D	3 500 ± 500	> 10 000	–	–	> 1 000	82 <sup>c</sup>
K38E/K124E/R127D	335 ± 30	1 000 ± 100	1.0 ± 0.2 × 10 <sup>5</sup>	19 ± 2 × 10 <sup>-3</sup>	190	95
K38E/K110E/K115E/K116E	350 ± 50	1 300 ± 110	1.1 ± 0.2 × 10 <sup>5</sup>	20 ± 3 × 10 <sup>-3</sup>	180	90
V3W	50 ± 10	80 ± 7				115
L2S/V3G	58 ± 7	180 ± 30				60 <sup>c</sup>
A18S/A19G/L20S	40 ± 6	150 ± 30				102
F24S	53 ± 7	110 ± 7				110

<sup>a</sup> gla minus factor Xa was used in the prothrombinase assay instead of factor Xa. <sup>b</sup> 300 mM NaCl was used during BIAcore experiments instead of 150 mM NaCl. <sup>c</sup> 20 μM fluorescent substrate was used instead of 1.5 μM to determine the specific activity of these two mutants (see Experimental procedures). IC<sub>50</sub> values and kinetic parameters are means ± SE of three independent experiments. For the specific activity, error in the numbers is ± 10%.

are exposed to solvent, the mutations are not expected to have a significant effect on the structure and stability of the proteins. This is likely to be the case as the mutant proteins were expressed in *E. coli* and refolded under conditions identical with those used for the wild-type hGIIA and similar overall yield were obtained. Moreover, for all the mutant proteins, the specific enzymatic activities measured with 1.5 μM fluorescent substrate were similar to that of wild-type hGIIA (Table 1), supporting the idea that these mutants are correctly refolded. For mutant K7E/K10E/K16E/R124D/K127E, a higher substrate concentration (20 μM) was used to obtain a specific activity similar to that of the wild-type enzyme (Table 1). This mutant has been shown to have a lower affinity for anionic phospholipid vesicles [35,40], and increased substrate concentrations are needed to reach an equivalent amount of enzyme bound to the substrate. The fact that the specific activity of this mutant is similar to that of the wild-type for high substrate concentrations argues for correct refolding of this protein. Even at 20 μM substrate, the mutant L2SV3G shows a slightly lower specific activity than that of the wild-type enzyme (Table 1), indicating that this mutant has a particularly low affinity for the fluorescent substrate. However, the fact that this mutant inhibits prothrombinase activity with similar efficiency to that of the wild-type enzyme argues for its correct refolding.

Table 1 shows that the mutants K53G/R54E, R132E and K100E inhibit prothrombinase activity with IC<sub>50</sub> values similar to that of the wild-type hGIIA. The IC<sub>50</sub> values for the mutants

K57N/K58L, K53E/R54E/R58E, K53E/R54E/K57E/R58E and R108E are slightly increased compared to that for the wild-type, and their kinetic constants (k<sub>on</sub>, k<sub>off</sub> and K<sub>d</sub><sup>app</sup>) for binding to factor Xa are similar to those determined for the wild-type (Table 1). In contrast, mutations of basic residues located on the IBS of hGIIA led to increases in the IC<sub>50</sub> values of fourfold (K74E/K87E/R92E, K124E/R127D, R7E/K10E/K16E), sevenfold (K38E/K124E/R127D and K38E/K110E/K115E/K116E) and 70-fold (R7E/K10E/K16E/K124E/R127D) (Table 1). The changes in IC<sub>50</sub> values correlated with increases in the K<sub>d</sub><sup>app</sup> values for binding to factor Xa, and the decrease in apparent affinity was found to be associated with higher k<sub>off</sub> values but unchanged, or slightly decreased, k<sub>on</sub> values (Table 1). In particular, the mutant R7E/K10E/K16E/K124E/R127D, which combines the removal of the basic charges of two clusters, K124/R127 and R7/K10/K16 (Fig. 2), shows a dramatic decrease in the prothrombinase effect. This large effect correlated with an increase of at least 200-fold in the K<sub>d</sub><sup>app</sup> value (Table 1). It should also be mentioned that single-site mutations of an individual basic cluster, for example K74E, K87E or R92E, produced only a modest change in the IC<sub>50</sub> values for prothrombinase inhibition. The effect of mutations on the ability of hGIIA to bind to factor Xa was stronger when investigated with SPR than when determined by prothrombinase assay, even though a good correlation between the relative activities in the two assays was observed. This may be because the direct binding of hGIIA to factor Xa was recorded



towards phosphatidylcholine vesicles [47], inhibits prothrombinase activity as efficiently as the wild-type hGIIA even in the presence of phospholipids.

### Effect of other mammalian and venom sPLA<sub>2</sub>s on prothrombinase activity

A few sPLA<sub>2</sub>s from snake and bee venoms were tested in prothrombinase assays. The basic sPLA<sub>2</sub> Ba IV from *B. asper* venom, a K-49 myotoxin devoid of catalytic activity [37], significantly inhibits prothrombinase activity in the presence or absence of phospholipid (Table 2). This result is in agreement with that obtained with H48Q hGIIA and suggests a phospholipid-independent mechanism of inhibition. On the other hand, the CM-III sPLA<sub>2</sub> from *N. mossambica mossambica* venom, although basic (pI = 9.9), and the neutral group III sPLA<sub>2</sub> from honey bee venom (*A. mellifera*) are unable to inhibit prothrombinase activity in the absence of phospholipids, whereas they are active in their presence (Table 2). This suggests that they exert their inhibitory effects through phospholipid hydrolysis.

We also analyzed whether other related mammalian sPLA<sub>2</sub>s (Fig. 3 and Table 2) inhibit prothrombinase activity. Rat and mouse group IIA sPLA<sub>2</sub>s (rGIIA and mGIIA), which are highly basic proteins like hGIIA, inhibit prothrombinase activity in the presence and absence of phospholipids, with IC<sub>50</sub> values close to those determined for hGIIA (Table 2). Human and rat group V (hGV and rGV) as well as mouse group IID (mIID) sPLA<sub>2</sub>s, which are all basic proteins, also inhibit the prothrombinase activity in the absence of phospholipids with similar IC<sub>50</sub> values (Table 2). In contrast, the less basic human group IB (pI = 8.7) and the weakly acidic bovine group IB (pI = 6) sPLA<sub>2</sub>s do not inhibit prothrombinase activity either in the absence or presence of phospholipids (Table 2 and data not shown). Finally, the human group X sPLA<sub>2</sub> (hGX), which is an acidic protein, efficiently inhibits prothrombinase activity in the presence of phospholipids (IC<sub>50</sub> = 35 nM), but is inactive in the absence of phospholipids, as observed for the bee venom sPLA<sub>2</sub> (Table 2). This suggests that these two sPLA<sub>2</sub>s exert an inhibitory effect by a mechanism different from that of hGIIA and the other basic sPLA<sub>2</sub>s, possibly through hydrolysis of procoagulant phospholipid as hGX and the bee venom sPLA<sub>2</sub>s are known to have high affinity for phosphatidylcholine vesicles [36].

## DISCUSSION

It has been previously reported that hGIIA inhibits *in vitro* the prothrombinase complex through its binding to factor Xa [30]. The present investigation further shows that hGIIA efficiently delays the formation of thrombin measured in human PRP, showing that the anticoagulant effect of hGIIA also occurs under experimental conditions close to physiological conditions. This *ex vivo* assay lacks endothelial cells and the associated extrinsic pathway, and is unable to mimick the accumulation of platelets and coagulation factors as it occurs *in vivo* at the thrombus clot. However, blood platelets and all plasma components of blood coagulation are present at physiological concentrations, and this *ex vivo* assay has been developed to detect hypercoagulability and hypocoagulability in patients and to monitor anticoagulant treatment [56–58]. Interestingly, catalytically inactive H48Q hGIIA induces an increase in the lag time of thrombin generation as efficiently as the wild-type enzyme. This demonstrates that the catalytic activity of hGIIA is not required for its anticoagulant effect even when natural phospholipids from plasma and activated

platelet membrane are present. Both wild-type and catalytically inactive hGIIA greatly delay the appearance of thrombin, but do not significantly decrease the amount of generated thrombin, in good agreement with an inhibitory mechanism based on the interaction of hGIIA with factor Xa. The function of hGIIA must therefore be to inhibit coagulation by delaying formation of the prothrombinase complex and thus the appearance of thrombin, rather than to inhibit the activity of the fully active prothrombinase complex which would decrease the amount of thrombin generated.

Such an anticoagulant effect of hGIIA is consistent with its inability to generate arachidonic acid from platelet membrane, and thus to participate in platelet activation [20,21]. It has been shown that hGIIA, in the presence of sphingomyelinase, is able to hydrolyse phospholipids from platelet-derived microvesicles [59]. These microvesicles support the coagulation cascade in addition to phospholipids from platelet membrane, and such an hydrolysis could lead to an anticoagulant effect [25–27]. However, it is unlikely that such microvesicular hydrolysis occurs during our *ex vivo* assay at a sufficient level to decrease significantly the procoagulant surface, because the catalytically inactive mutant (H48Q) shows the same efficiency as the wild-type hGIIA in inhibiting thrombin generation in PRP. Furthermore, hGIIA has been shown to increase the generation of prostacyclin by vascular endothelial cells [60], which is an efficient inhibitor of platelet activation. The occurrence of such an effect *in vivo* would amplify the anticoagulant effect of hGIIA. We were unable to investigate whether this additional anticoagulant effect does occur because of the absence of endothelial cells in our *ex vivo* assay.

It has been established that the levels of hGIIA in serum greatly increase from 0.35 nM to 0.6 μM under various inflammatory conditions [61,62]. We report here that hGIIA inhibits with an IC<sub>50</sub> value of 80 nM the prothrombinase activity measured *in vitro* in the presence of phospholipids. Furthermore, a K<sub>d</sub> value of 230 nM was previously determined by isothermal microcalorimetry for the interaction of hGIIA with factor Xa, which is a more relevant value than the K<sub>d</sub><sup>app</sup> value determined by SPR, where one of the two components (factor Xa) is immobilized [30]. In any case, these IC<sub>50</sub> and K<sub>d</sub> values appear consistent with a potential localized and/or systemic anticoagulant role of hGIIA during various physiological or pathological states. The inhibition of thrombin generation in PRP by hGIIA was significant for concentrations around 1 μM, i.e. at values slightly higher than the IC<sub>50</sub> and K<sub>d</sub> values measured *in vitro*. This difference may be due to the large increase in blood coagulation efficiency observed with activated platelets in thrombin-generation experiments, in which high factor V/factor Va concentrations are present and compete for hGIIA/factor Xa binding, as well as to the presence of natural phospholipids which may slightly modify the K<sub>d</sub> value of the hGIIA–factor Xa interaction. This value is slightly higher than the levels of hGIIA determined in serum, i.e. in the systemic circulation. However, it is well established that blood platelets secrete large amounts of hGIIA upon activation [17–19]. Therefore, the hGIIA secreted from platelets will participate, in addition to the hGIIA coming from the systemic circulation, to reach high local concentrations during thrombolytic events [9,59]. It has been shown that hGIIA is adsorbed at the platelet surface during secretion, and therefore will not be diluted into the systemic circulation [63]. In agreement with this, low micromolar concentrations of hGIIA have been locally described in synovial fluids from patients with various inflammatory diseases [64], ascitic fluid [65], seminal fluid [66] and tears [67].

This study also shows that several surface-exposed basic residues of hGIIA are key elements in the interaction with factor Xa. We were unable to detect substantial changes in the affinity for factor Xa upon mutation of residues K53, R54, K57 and R58, even when all four were simultaneously replaced by negatives charges. This was surprising as it has been reported that the replacement of Lys57 by glutamine causes a 10-fold reduction in antiprothrombinase activity of hGIIA [24], and that a synthetic peptide comprising residues 51–74 of hGIIA inhibits prothrombinase activity [30]. The present investigation indicates that residues 53–58 of hGIIA do not play a major role in the interaction with factor Xa. In contrast, clusters of basic residues located on the IBS probably play an important role in the interaction of hGIIA with factor Xa, as mutations of these residues lead to a significant reduction in the ability of hGIIA to inhibit prothrombinase activity and to bind to factor Xa. This suggests that electrostatic interactions probably play a crucial role in the hGIIA–factor Xa interaction, as also supported by the observation that increased salt concentrations completely abolished the binding of hGIIA to factor Xa (Table 1). The affinity of hGIIA for factor Xa is abolished when two distant basic clusters of the IBS are simultaneously replaced by acidic clusters, suggesting that the surface of the factor Xa–hGIIA interaction is rather large and composed of several basic residues. It also explains why a single-site mutation only induces a modest change in the ability of hGIIA to inhibit prothrombinase activity and bind to factor Xa, whereas pronounced changes are obtained with multiple mutations of basic residues in the same cluster. Furthermore, a non-specific effect due to a change in the overall basicity of the hGIIA seems unlikely, as the mutant K53E/R54E/K57E/R58E binds to factor Xa with an affinity similar to that of wild-type hGIIA. In addition, mutations of surface-exposed hydrophobic residues located on the IBS of hGIIA (Fig. 2) failed to interfere with the ability to inhibit prothrombinase activity in the absence of phospholipids (Table 1), suggesting that these residues do not make significant contact with factor Xa. However, we cannot exclude the possibility that other hydrophobic residue(s), not tested in this study, may be involved in the interaction with factor Xa.

Although the anticoagulant activity of venom sPLA<sub>2</sub>s is well established [31–34], some controversy exists in the literature over the mechanism. Some studies suggest that the sPLA<sub>2</sub> catalytic activity is important [33,68], whereas others support the notion that the catalytic activity is not required [34,69,70]. Our present investigation suggests that both hypotheses may be correct, and that the mechanism of inhibition depends on the particular sPLA<sub>2</sub>. For instance, we show here that the catalytically inactive basic venom sPLA<sub>2</sub>, Ba IV, can inhibit prothrombinase activity in the absence of phospholipids, suggesting that it acts through direct binding to factor Xa. Similar efficiency to inhibit prothrombinase was observed with the basic subunit of crotoxin (data not shown). These observations are in good agreement with the recent report of a direct interaction between the basic sPLA<sub>2</sub> from *Naja nigricollis* and bovine factor Xa [71]. It is thus likely that some basic venom sPLA<sub>2</sub>s inhibit blood coagulation through direct binding to factor Xa, rather than, or in addition to, a hydrolytic action on phospholipids. On the other hand, our data suggest that other venom sPLA<sub>2</sub>s, such as CM-III and bee venom sPLA<sub>2</sub>s, inhibit prothrombinase activity through phospholipid hydrolysis (Table 2).

As the phospholipid-independent anticoagulant effect of hGIIA appears to be relevant for some basic venom sPLA<sub>2</sub>s, we investigated whether such an effect could also be extended to the recently cloned mammalian sPLA<sub>2</sub>s. Mammalian rGIIA,

mGIIA, hGV, rGV and mGIID sPLA<sub>2</sub>s were found to inhibit prothrombinase activity in the absence of phospholipids with an efficiency similar to that of hGIIA, suggesting that they may also act through direct binding to factor Xa. These sPLA<sub>2</sub>s are all basic proteins, as noted above for venom sPLA<sub>2</sub>s. However, the overall basicity of the molecule is not sufficient as the basic CM-III sPLA<sub>2</sub>s (Table 2) and the basic AmtxA from *Vipera ammodytes ammodytes* venom (data not shown) are devoid of inhibitory effect in the absence of phospholipids. The structure–function study presented here for hGIIA indicates that several basic residues located on the IBS are key elements for the interaction with factor Xa and prothrombinase inhibition. Amino-acid alignment of hGIIA with other mammalian sPLA<sub>2</sub>s shows that several of these residues are conserved between basic sPLA<sub>2</sub>s active on prothrombinase via a phospholipid-independent mechanism (Fig. 3). In particular, rGIIA and mGIIA share with hGIIA residues K16, K38, K74, K110, K116, and R127 or K127, shown here to be important basic residues for the interaction with factor Xa. Interestingly, except for K110, all these basic residues are conserved in mGIID, a basic sPLA<sub>2</sub> that presents an IC<sub>50</sub> on prothrombinase activity in the absence of phospholipids close to that of the hGIIA. The basic residues of hGIIA involved in the interaction with factor Xa are less conserved in mGV or rGV, only K16, K38 and K74 or R116 being present. In this case, the loss of one or some defined basic residue(s) may account for the slightly lower efficiency of hGV in inhibiting prothrombinase activity ( $150 \pm 10$  nM compared with  $50 \pm 6$  nM for hGIIA). It is thus likely that the precise interaction site of the group V sPLA<sub>2</sub>s is not exactly the same as that of hGIIA. The basic residues of hGIIA involved in the interaction with factor Xa are all located on the IBS but are distributed throughout the primary sequence of the protein (Figs 2 and 3). It is therefore possible that basic residues located at different positions on the primary sequence of other sPLA<sub>2</sub>s are involved in the formation of similar basic cluster patterns, and molecular modeling will be useful in a more detailed investigation of this hypothesis. Neutral or acidic mammalian sPLA<sub>2</sub>s such as hGIB and hGX were unable to inhibit prothrombinase activity in the absence of phospholipids, further supporting the critical role of basic residues in the phospholipid-independent antiprothrombinase activity. In agreement, these two sPLA<sub>2</sub>s do not share with hGIIA most of the basic residues K16, K38, K74, K110, K116 and R127 (Fig. 3).

This study indeed shows that, in addition to hGIIA, three other mammalian sPLA<sub>2</sub>s are able to inhibit blood coagulation, either by acting through direct binding to factor Xa (hGV, rGV and mGIID) or by phospholipid hydrolysis (hGX). This raises the question of the circulating levels of these sPLA<sub>2</sub>s in blood and of their physiological or pathophysiological roles in thrombolytic events. So far, data on the circulating levels of GIID, GV, or GX are lacking, even though the presence of GV and GX in blood is likely as the former is detected in macrophages and mast cells, and the latter in peripheral blood leukocytes [4,5]. It may be possible that the levels of anticoagulant sPLA<sub>2</sub>s in blood are higher than expected and depend on various physiopathological conditions in which one or more of these sPLA<sub>2</sub>s can be secreted.

In conclusion, our results show that hGIIA probably exerts an anticoagulant effect during physiological and/or pathological situations. This effect occurs through direct binding to factor Xa, via basic residues located on the IBS of hGIIA, and does not require sPLA<sub>2</sub> catalytic activity. This study also indicates that several other mammalian and venom sPLA<sub>2</sub>s inhibit prothrombinase activity by a mechanism similar to that of

hGIIA, whereas others act on phospholipids. This leads to the hypothesis that various mammalian sPLA<sub>2</sub>s may act in concert to inhibit blood coagulation, through either binding to factor Xa or an action on procoagulant phospholipids.

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