Modulation of Factor V Levels in Plasma by Polymorphisms in the C2 Domain

Daniela Scanavini, Domenico Girelli, Barbara Lunghi, Nicola Martinelli, Cristina Legnani, Mirko Pinotti, Gualtiero Palareti, Francesco Bernardi

Objective—Functional polymorphisms contributing to coagulation factor levels are preferential markers for association studies aimed at identifying prothrombic genetic components.

Methods and Results—Factor V (FV) microsatellite genotypes were found to be associated with FV levels ($P=0.003$). Single nucleotide polymorphisms analysis and sequencing of the promoter and of coding regions identified two polymorphisms (Met2120Thr, Asp2194Gly) present in 20% of the population ($n=1013$) that are responsible for genotype–phenotype associations. The effect of the Met2120Thr polymorphism, both in plasma (mean reduction of FV level in the heterozygous condition: 25%) and in recombinant FV studies (34% reduction), was comparable to that of the Asp2194Gly change (20% and 34%, respectively). The study of 10 subjects with a rare genotype indicated that the Asp2194Gly substitution is the functional determinant of the reduced FV levels associated with the FVHR2 haplotype. Among Leiden carriers, the doubly heterozygous condition for FV2120Thr was found to be associated with a significantly increased activated protein-C resistance (APCR) ($P<0.05$), and the doubly heterozygous condition for FV2194Gly was found to be more frequent ($P=0.009$) in symptomatic than in asymptomatic subjects.

Conclusions—Extensive analysis of FV polymorphisms indicated that changes in the C2 domain modulate FV levels and might increase APCR and thrombotic risk in FV Leiden carriers through a pseudohomozygous mechanism. (Arterioscler Thromb Vasc Biol. 2004;24:200-206.)

Key Words: factor V levels ■ functional polymorphisms ■ FVHR2 haplotype ■ recombinant FV ■ APCR resistance

Polymorphisms contributing to modulate factor levels might constitute preferential markers for association studies aimed at identifying prothrombic or protective genetic components. Among the coagulation factors, factor V (FV) plays a pivotal role at the crossroads of procoagulant and anticoagulant pathways, acting as a cofactor in prothrombin activation and in activated factor VIII (FVIII) inactivation. A complex relationship between plasmatic FV levels and thrombosis might be present; high FV coagulant levels have been found to be an independent risk factor for myocardial infarction, whereas no significant association was found between FV antigen level and thrombotic risk. Moreover, low FV levels are associated with a reduced activated protein-C (APC) cofactor activity in the inactivation of the FVIIIa, which could be responsible for an APC-resistant phenotype. In particular, low FV levels in carriers of FV Leiden mutation result in a pseudohomozygous state of APCR resistance (APCR). Although FV genetic components of APCR have been extensively investigated, no systematic analysis of the relationship between FV gene variation and FV levels has been conducted. The His1299Arg polymorphism, which marks a FV gene haplotype (FVHR2) predicting several amino acid substitutions in the A2, B, A3, and C2 domains, has been associated with reduced FV activity and antigen levels. Because the increased risk for venous thrombosis conferred by the FVHR2 haplotype is still a matter of debate, markers of this haplotype are currently investigated in several laboratories.

FV expression studies have indicated that the Asp2194Gly change in the C2 domain, among the several polymorphisms of the FVHR2 haplotype, is responsible for significantly reduced FV levels in conditioned media and for reduced secretion rate. Linkage of markers in the haplotype has prevented validation in vivo of this functional candidate. We investigated, in a large cohort of subjects, the presence of frequent genetic components of plasma FV levels. The relationship of candidate single nucleotide polymorphisms (SNPs) with APCR and venous thrombosis was then evaluated in FV Leiden carriers. Finally, the functional consequences of SNPs were investigated through expression of recombinant FV.

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409 G/C (Asp79His)  
1691 G/A (Arg506Gln)  
4070 A/G* (His1299Arg*)

EX 3  EX 10  EX 13

Intron 11
GT repeat (12R*-16R)

EX 16  EX 24  EX 25

Intron 22
-20 C/A*

C2 domain

FV gene localization of the microsatellite and SNPs investigated in this study. Selected exons are reported together with nucleotide and amino acid changes. Polymorphisms modulating FV levels are reported in bold. The alleles characterizing the FVHR2 are indicated by an asterisk.

Methods

Population Studies

In this study, 1013 subjects (769 males and 244 females, mean age: 60.1±10.6 years) living in Verona were enrolled in the frame of a regional cardiovascular disease survey, characterized for FV levels, and genotyped for FV polymorphisms.

One hundred twenty-two carriers of FV Leiden mutation (42 males and 80 females, mean age at the first thrombotic event: 35.9±12.8 years) were characterized for APC ratio and genotyped for FV polymorphisms. They were recruited in a thrombophilia screening and met the following criteria: (1) objectively confirmed leg deep venous thrombosis and/or pulmonary embolism; (2) blood sampled >3 months after the last thrombotic event and >3 weeks after anticoagulation withdrawal; and (3) normal liver function and no evidence of autoimmune or neoplastic disease.

Two-hundred ninety unrelated carriers with FV Leiden mutation diagnosed between September 1994 and January 2001 in the Angiology Department of Bologna University were investigated for the association of FV polymorphisms with venous thrombosis. Subjects were selected and matched as previously reported.54 All subjects were of Italian origin and none had other known thrombophilic conditions (such as antithrombin, protein C and protein S deficiency, or G20210A mutation of the prothrombin gene). Of these subjects, 145 were unrelated patients who experienced an objectively confirmed venous thromboembolic episode (VTE) (49 males and 96 females, mean age at first VTE: 35.5±11.6 years). The 145 unrelated subjects were still asymptomatic for VTE at presentation (49 males and 96 females, mean age at first VTE: 35.9±12.8 years) were characterized for APC ratio and genotyped for FV polymorphisms. They were recruited in a thrombophilia screening and met the following criteria: (1) objectively confirmed leg deep venous thrombosis and/or pulmonary embolism; (2) blood sampled >3 months after the last thrombotic event and >3 weeks after anticoagulation withdrawal; and (3) normal liver function and no evidence of autoimmune or neoplastic disease.

Either written or verbal informed consent was obtained from all subjects. The study was performed according to the Helsinki protocol and approved by our institutional review board.

DNA Studies

FV markers investigated in this study are shown in Figure 1 (numbering in accordance with that of Jenny et al24). Automated sequencing was performed with the ABI Prism 377 DNA Sequencer (PE Applied Biosystems, Foster City, CA). Primer sequences and polymerase chain reaction (PCR) conditions are listed in Table I (available online at http://atvb.ahajournals.org).

The -426G/A polymorphism35 was investigated by MvaI digestion of the PCR fragment PromF–PromR. The 409G/C (Asp79His) polymorphism36 was detected by using the FIVS3 primer and a mutagenized reverse primer (5'-GGATGGATGCTCAAGGGCTGATGAT-3'), introducing a MboI restriction site in the wild-type (G) allele. Genotyping for the FV 1691G/A (Arg506Gln), 4070A/G (His1299Arg), 5380A/G (Met1736Val) polymorphisms and for the intron 11 microsatellite marker was performed as previously reported.13,18,20,21 The C/A polymorphism36 in the intron 22 (~20 bp from exon 23; nt 31701 at accession number Z99572) was identified by HpyCH4IV digestion of the PCR fragments FIVS23–RIVS23. The 6533T/C (Met2120Thr) change35 was screened by amplification of a 316-bp fragment with the primers FIVS24–RIVS24, followed by HpyCH4IV digestion.

A mutagenized primer (5'-TCTGTCCTCTTGAAACGATGTGAACAGCTATACC-3'), introducing a RsaI restriction site in the rare (G) allele, was designed to detect the 6755A/G (Asp2194Gly) change. A multiplex PCR (30 seconds at 95°C, 30 seconds at 56°C, 1 minute at 72°C, 30 cycles) was set-up for the analysis of His1299Arg and Asp2194Gly polymorphisms, which were both detectable through RsaI digestion.

FV Activity

Plasma FV activity was measured by a one-stage clotting method (Thromborel S and FV depleted plasma, Dade Behring, Marburg, Germany). APC ratio was measured in accordance with the methods of de Ronde and Bertina.38

The activity of recombinant FV molecules was evaluated as thrombin generation in a FV depleted plasma system diluted 1:6 in medium containing recombinant FV and incubated at 37°C for 3 minutes with Innovin (Dade Behring) to trigger coagulation. Aliquots of the reaction were then quenched with 12 mmol/L EDTA. Thrombin was quantified by estimating its activity toward 250 μmol/L thrombin fluorogenic substrate (ICN Biomedicals, Costa Mesa, CA). Fluorescence (360 nm excitation, 465 nm emission) was monitored over time on a Spectrafluor Plus microplate reader (Tecan, Salzburg, Austria). The initial rates, expressed as relative fluorescence units (RFU) per second, were derived from thrombin generation curves. Rates obtained for mutant proteins were compared with those of the wild-type protein by Student’s t test, and the activity of mutants was expressed as percentage of wild type.
TABLE 1. FV Activity Distribution in Subjects Grouped for IVS11 Microsatellite Marker

<table>
<thead>
<tr>
<th>IVS 11 FV Genotypes</th>
<th>Subjects</th>
<th>%</th>
<th>FV Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>15R/15R</td>
<td>38</td>
<td>9.5</td>
<td>140.3 ± 31.8*</td>
</tr>
<tr>
<td>14R/15R</td>
<td>119 (1)</td>
<td>29.6</td>
<td>138.7 ± 32.9*</td>
</tr>
<tr>
<td>12R/15R</td>
<td>35 (14)</td>
<td>8.7</td>
<td>137.3 ± 41.8</td>
</tr>
<tr>
<td>13R/14R</td>
<td>25 (2)</td>
<td>6.2</td>
<td>135.7 ± 43.9</td>
</tr>
<tr>
<td>14R/14R</td>
<td>89 (1)</td>
<td>22.1</td>
<td>134.7 ± 37.2</td>
</tr>
<tr>
<td>13R/15R</td>
<td>16</td>
<td>4.0</td>
<td>134.1 ± 39.2</td>
</tr>
<tr>
<td>12R/13R</td>
<td>7 (2)</td>
<td>1.7</td>
<td>130.6 ± 23.0</td>
</tr>
<tr>
<td>12R/16R</td>
<td>2 (1)</td>
<td>0.5</td>
<td>127.5 ± 44.5</td>
</tr>
<tr>
<td>12R/14R</td>
<td>51 (36)</td>
<td>12.7</td>
<td>116.0 ± 30.0</td>
</tr>
<tr>
<td>14R/16R</td>
<td>1</td>
<td>0.2</td>
<td>115.0</td>
</tr>
<tr>
<td>12R/12R</td>
<td>15 (13)</td>
<td>3.7</td>
<td>113.8 ± 31.0</td>
</tr>
<tr>
<td>13R/13R</td>
<td>4</td>
<td>1.0</td>
<td>110.8 ± 21.7</td>
</tr>
<tr>
<td>All genotypes</td>
<td>402 (70)</td>
<td>100.0</td>
<td>133.1 ± 35.7</td>
</tr>
</tbody>
</table>

12R to 16R, alleles of IVS11 microsatellite marker. 

n indicates number of subjects; parenthesis, number of carriers of at least one 6755G (2194Gly) allele; %, percentage of subjects.

FV activity was measured as % of PNP (mean ± SD).

ANOVA (F = 3.10, P = 0.003) was conducted for genotypes present in at least 15 subjects.

*Significantly different from the 12R/14R group (Bonferroni t test, P < 0.05)

FV Antigen

FV antigen was determined using a two-site immunoassay (ZYMUTEST Factor V kit; HYPHEN BioMed, Andrésy, France).

Statistical Analysis

Statistical analyses were performed with SPSS 10.0 statistical package (SPSS, Chicago, IL). FV activity distributions in subjects grouped for genotypes were compared by the ANOVA with Bonferroni post-hoc comparison of the means. Differences between recombinant FV levels were assessed by Student’s t test. FV polymorphism distributions in FV Leiden carriers were compared in the groups of patients and asymptomatic subjects by χ² analysis. The Hardy–Weinberg equilibrium of genotype frequencies was also tested by χ² analysis.

RESULTS

Microsatellite Analysis

To trace multiple genetic components of FV level modulation in plasma, as a preliminary step we genotyped for a genetically stable microsatellite marker. 402 subjects belonging to a larger enrolled group (1013 subjects) were included. This polymorphism is characterized by 5 alleles (12 to 16 repeats, alleles 12R–16R) and produced 12 genotype groups, which are ranked in Table 1 for mean FV levels in plasma. A significant association of FV levels with genotypes (ANOVA, F = 3.10, P = 0.003) was found, and differences between groups reached statistical significance (see Table 1).

Among homozygous groups in Table 1, the 15R homozygotes are located at the top (higher levels), whereas 12R and 13R homozygotes are listed at the bottom (lower levels). In addition, the genotypes heterozygous for the 15R allele are preferentially distributed among subjects with higher mean FV levels, whereas 12R heterozygotes, with the noticeable exception of the double heterozygotes 12R/15R, are preferentially distributed among subjects with lower mean FV levels. These observations provided evidence for the presence of frequent FV genetic components modulating FV levels.

SNPs Analysis

Because of the intronic localization of the microsatellite marker, which does not make its alleles plausible candidates to produce functional changes, subjects homozygous for 15R, 13R, and 12R alleles were screened for SNPs (Figure 1) in coding regions. 18,19,39,40

SNP analysis of the 15R homozygotes (n = 38) indicated that the 5380A/G change (Met1736Val) in the exon 16 was over-represented (22/38 “GG” homozygotes; G allele frequency: 75%) among these subjects. As comparison, the G allele frequency in the whole population was 40%. However, no significant differences in FV levels were observed in subject grouped for the 1736Met/Met, 1736Val/Val, and 1736Met/Val genotypes.

Heterogeneous SNP genotypes were found in the small group of 13R homozygotes (n = 4), which prevented further analysis. Among the 12R homozygotes (n = 15), 7 were carriers and 6 were homozygotes for the 4070A/G (His1299Arg) substitution, which marks the FVHR2 haplotype.12,22,29

Because the IVS11 microsatellite marker is genetically stable,18 it enables us to detect ancient genetic components such as the FVHR2,21 which is still associated with the 12R allele.

The Asp2194Gly polymorphism in the C2 domain, which is suggested as a functional candidate of the FVHR2 haplotype by expression studies,12,33 was further investigated. To favor the detection of the rare subjects characterized by the FVHR2 haplotype with a wild-type (2194Asp) C2 domain,22 genotyping was extended to a larger sample (1013 subjects; Table 2), which included the 402 subjects investigated in Table 1. A multiplex PCR system was designed to detect both
His1299Arg and Asp2194Gly polymorphisms. One hundred thirty-four subjects heterozygous for the His1299Arg change were also carriers of the Asp2194Gly change. However, 10 subjects were found to be homozygous for Asp at position 2194 and thus were carriers of a FVHR2 variant. In this FV gene, linkage disequilibrium between the 1299Arg and the 2194Gly codons located in the exon 13 and 25, respectively, has been interrupted by recombination, as supported by genotyping for the −20 C/A polymorphism in intron 22. We found that the A allele belongs to the FVHR2 haplotype (unpublished observation). The 1299His-Ar-2194AspAsp subjects (n=10) were found to be homozygous for the CC (wild-type) genotype, showing that the recombination occurred downstream to IVS22.

The 1299His-Ar-2194AspAsp genotypes offer the opportunity to test the contribution in plasma of the Asp2194Gly change downstream to IVS22.

We found that the A allele belongs to the FVHR2 haplotype (wild-type) genotype, showing that the recombination occurred downstream to IVS22.

The 1299His-Ar-2194AspAsp genotypes offer the opportunity to test the contribution in plasma of the Asp2194Gly change to the FV phenotype. FV activity (Table 2) was significantly (P<0.05) lower in the 1299HisArg-2194AspAsp subjects (n=134) than in homozygotes for the wild-type FV gene (1299His-His-2194AspAsp, n=812), whereas the rare (n=10) 1299His-Ar-2194AspAsp subjects had FV activity similar to that of the wild-type group.

**Search of New Determinants by Nucleotide Sequencing**

When the 2194Gly carriers distributed among the genotype groups in Table 1 (70 of 402, mean FV:C: 110.9±26.3 SD) were excluded from the ANOVA, significant differences in FV levels were no longer detectable (F=0.73; P=0.664). This finding indicates that the Asp2194Gly change was the main genetic component underlying FV level differences detected by the microsatellite marker, thus preventing further genetic studies based on this approach.

To find new genetic components of FV levels not identified by the microsatellite analysis, 20 subjects with the lowest FV levels (first and second percentiles, FV:C 38.5±SD), this marker was not further investigated. A total of 5.4 kb of FV genomic DNA was sequenced using the PCR conditions and the primers reported to 287). We only detected a previously reported polymorphism (−426 G/A) that was extensively genotyped. Heterozygotes (n=121) for this marker showed mean FV levels (127.7±4.2 SD) and 97His/His (genotypic frequency 11.7%, mean FV:C 117.0±34.0 SD). This marker was not further investigated.

Sequence the microsatellite marker region was also used to investigate the presence of polymorphic candidates to increase plasma FV levels in 10 subjects with FV levels in the highest percentiles (90 and 100 percentiles, FV:C range: 183 to 287). We only detected a previously reported polymorphism (−426 G/A) that was extensively genotyped. Heterozygotes (n=121) for this marker showed mean FV levels (127.7±4.2 SD) and 97His/His (genotypic frequency 0.3%, mean FV:C 117.0±34.0 SD). This marker was not further investigated.

**Genotyping of Subjects Carrying FV Leiden Mutation**

In the doubly heterozygous condition with FV Leiden, the Met2120Thr and Asp2194Gly changes could produce, through a partial pseudohomozygous mechanism, a relative

**TABLE 3. APC Ratio in Thrombotic FV Leiden Carriers**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>%</th>
<th>APC Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV Leiden/Wt</td>
<td>99</td>
<td>81.2</td>
<td>0.55±0.07</td>
</tr>
<tr>
<td>FV Leiden/2120Thr</td>
<td>7</td>
<td>5.7</td>
<td>0.48±0.05*</td>
</tr>
<tr>
<td>FV Leiden/HR2</td>
<td>16</td>
<td>13.1</td>
<td>0.52±0.06</td>
</tr>
<tr>
<td>All genotypes</td>
<td>122</td>
<td>100.0</td>
<td>0.54±0.07</td>
</tr>
</tbody>
</table>

FV haplotypes are separated by a slash.

FV Leiden indicates haplotype 506Gln 1299His 2120Met 2194Asp; Wt, haplotype 506Arg 1299His 2120Met 2194Asp; 2120Thr, haplotype 506Arg 1299His 2120Thr 2194Asp; HR2, haplotype 506Arg 1299Arg 2120Met 2194Gly.

n and % indicate number and percentage of subjects, respectively.

APC ratio was reported as mean±SD.

ANOVA, F=4.44, P=0.014

*Significantly different from the Wt group (Bonferroni test, P<0.05)

studies, to represent a neutral polymorphism was detected in 5 subjects by sequencing of the exon 3. Screening for this polymorphism was extended to the whole population. We did not find significant differences (ANOVA, F=2.10, P=0.123) in FV levels among genotype groups: 79Asp/Asp (Wt) (genotypic frequency 88%; mean FV:C 135.1±38.5 SD), 79Asp/His (genotypic frequency 11.7%, mean FV:C 127.8±42.9 SD) and 97His/His (genotypic frequency 0.3%, mean FV:C 117.0±34.0 SD). This marker was not further investigated.

**TABLE 4. FV Polymorphisms and Venous Thrombosis in 290 FV Leiden Carriers**

<table>
<thead>
<tr>
<th>FV Polymorphisms</th>
<th>Genotypes</th>
<th>Symptomatic Subjects (n=145)</th>
<th>Asymptomatic Subjects (n=145)</th>
</tr>
</thead>
<tbody>
<tr>
<td>653S/T (C,Met2120Thr)*</td>
<td>TT</td>
<td>135</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>6755A/G (Asp2194Gly)†</td>
<td>AA</td>
<td>127</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>18</td>
<td>5</td>
</tr>
</tbody>
</table>

n indicates number of subjects.

*χ²=2.899 (P=0.089)

†χ²=6.800 (P=0.009)
increase in plasma levels of APC resistant molecules. A significant (ANOVA, F=4.44, P=0.014) contribution to APCR of these polymorphisms was detectable in 122 unrelated thrombotic patients carriers of FV Leiden (Table 3). Seven were doubly heterozygous for the FV Leiden and the 2120Thr allele and showed a significant (P<0.05) decrease in APCR of these polymorphisms as compared with simple FV Leiden carriers (n=99). Although slightly decreased, APCR ratios in the 16 doubly heterozygous for FV Leiden and the 2194Gly allele did not significantly differ from that of the 2194Asp and FV Leiden carriers.

To investigate the contribution of FV polymorphisms to venous thrombosis, we genotyped for the Met1736Val and Asp2194Gly changes in 290 carriers of FV Leiden. Among them, 145 were VTE patients and 145 were still asymptomatic, selected because they were the same age or older than symptomatic patients, and favored the detection of genetic components modulating the risk of thrombosis in FV Leiden carriers. FV genotypes distribution (Table 4) showed that the Asp2194Gly polymorphism was more frequent (χ²=6.800, P=0.009) among VTE patients. The 2120Met carriers were also more represented among patients, but the difference did not reach statistical significance (χ²=2.899, P=0.089).

Recombinant FV Expression

To corroborate the observations indicating positive association between markers and FV levels, we evaluated in vitro the functional impact of thromine at position 2120 of FV C2 domain. Expression in eucaryotic COS-7 cells of the recombinant 2120Thr-FV was compared with that of Wt

<table>
<thead>
<tr>
<th>TABLE 5. FV Levels in Medium of Recombinant FV</th>
</tr>
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<tbody>
<tr>
<td>FV Mutants, 2120Met, 2194Asp (Wt)</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>1299His, 2120Met, 2194Asp</td>
</tr>
<tr>
<td>1299His, 2120Thr, 2194Asp</td>
</tr>
<tr>
<td>1299His, 2120Met, 2194Gly</td>
</tr>
</tbody>
</table>

n indicates number of independent transfections.

FV activity was expressed as % of Wt; FV antigen was expressed as ng/mL (mean±SD).

t-tests: P<0.001; †P=0.043; §P=0.006; P=0.030

extensive investigation of a genetically stable microsatellite marker and several SNPs. Moreover, sequencing of the promoter region, and of a large portion of coding regions, was performed in selected subjects. We have combined genotyping in a relatively large cohort of subjects characterized for FV levels with expression of recombinant FV carrying candidate mutations. This approach provides us with valuable information to further investigate the association between clinical phenotypes and FV genotypes.

The population in this study was enrolled in a regional study of coronary heart disease and consequently is characterized by older age than subjects contributing to the normal pooled plasma who were selected among several age classes. The observation that FV levels increase with age could partially explain the high mean FV levels observed in our sample.

Several microsatellite genotype groups, characterized by the highest FV levels, ie, the first 6 groups listed in Table 1, showed very similar mean FV activity values, which does not suggest the presence of frequent underlying FV mutations leading to significant differences in FV expression. The possibility that promoter mutations genetically linked to frequent microsatellite alleles would have eluded detection was ruled out by nucleotide sequencing in the 5' region, which was performed in subjects selected for FV levels. The sequencing approach, extended to 80% of coding regions, did not reveal any new gene variation, and genotyping for polymorphisms in the 5' region (−426G/A) and in exon 16 (Met1736Val) did not show association with high FV levels. The secretion rate of the 1736Val FV has been previously found to be indistinguishable from that of wild-type FV. Taken together, these observations suggest that the control of FV gene expression leading to high FV levels is likely to be exerted mainly by environmental factors and perhaps by genetic components not linked to FV gene, as recently reported for other coagulation parameters such as APC resistance.

However, microsatellite analysis clearly suggested that frequent gene variations within FV contributes to the production of the relatively lower FV levels, and SNP analysis indicated that an important contribution to the modulation of FV levels was conferred by two polymorphisms, both located in the C2 domain. The quantitative impact of the Met2120Thr polymorphism in lowering FV levels was detectable both in plasma (25% mean reduction in the heterozygous condition) and in FV expression studies (34% reduction), and it was comparable to that of the Asp2194Gly change (20% and 34%, respectively).

The C2 domain contains essential binding sites of FVa for membranes, which might suggest that these polymorphisms result in reduced FV activity through decreased interaction of mutant FV with phospholipid membranes. However, we detected a parallel reduction in activity and antigen levels in media of cells expressing the recombinant FV mutants, which suggests the presence of mild quantitative defects. Decreased FV antigen levels were also found in plasma of carriers of both polymorphisms (D.G., unpublished results).

Discussion

To investigate FV gene variation contributing to FV levels in plasma, we have used a systematic approach based on an extensive investigation of a genetically stable microsatellite marker and several SNPs. Moreover, sequencing of the promoter region, and of a large portion of coding regions, was performed in selected subjects. We have combined genotyping in a relatively large cohort of subjects characterized for FV levels with expression of recombinant FV carrying candidate mutations. This approach provides us with valuable information to further investigate the association between clinical phenotypes and FV genotypes.

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The C2 domain contains essential binding sites of FVa for membranes, which might suggest that these polymorphisms result in reduced FV activity through decreased interaction of mutant FV with phospholipid membranes. However, we detected a parallel reduction in activity and antigen levels in media of cells expressing the recombinant FV mutants, which suggests the presence of mild quantitative defects. Decreased FV antigen levels were also found in plasma of carriers of both polymorphisms (D.G., unpublished results).
The Asp2194Gly substitution has previously been suggested as a functional candidate for the effect of the FVHR2 haplotype on FV levels, either by the study of a small group of FVHR2 homozygotes or by expression studies. The presence in our population study of 10 subjects carrying a rare, naturally occurring FVHR2 variant lacking the Asp2194Gly change provided strong evidence, based on an in vivo model, for the functional role of the 2194Gly FV variant in lowering FV levels. The Asp2194Gly substitution, among the several amino acid changes characterizing the FVHR2 haplotype, is the functional determinant of this phenotype.

These genetic markers of relatively reduced FV levels were present in approximately one fifth of the population and provide examples of frequent and subtle inherited components that could interact with major thrombophilic mutations. The Met210Thr and Asp2194Gly polymorphisms, associated with a 25% reduction of FV levels, could produce, in the doubly heterozygous condition with FV Leiden, an approximately 2-fold relative excess of FV Leiden molecules in plasma, which is a partially pseudohomozygous condition. Accordingly, we found that the 2120Thr allele was associated with a 25% reduction of FV levels, could produce, in the in vivo model, for the functional role of the 2194Gly FV variant 

The relationship between carrying these polymorphisms and thrombosis was also investigated in carriers of FV Leiden by comparing genotype distribution in VTE patients and in asymptomatic subjects. This analysis further supported a prothrombic role of the Asp2194Gly change and of the Asp2194Gly change provided strong evidence, based on an in vivo model, for the functional role of the 2194Gly FV variant 

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