

HEMOSTASIS, THROMBOSIS, AND VASCULAR BIOLOGY

ERGIC-53 Gene Structure and Mutation Analysis in 19 Combined Factors V and VIII Deficiency Families

By William C. Nichols, Valeri H. Terry, Matthew A. Wheatley, Angela Yang, Ariella Zivelin, Nicola Ciavarella, Caterina Stefanile, Tadashi Matsushita, Hidehiko Saito, Norma B. de Bosch, Arlette Ruiz-Saez, Argimiro Torres, Arthur R. Thompson, Donald I. Feinstein, Gilbert C. White, Claude Negrier, Christine Vinciguerra, Melih Aktan, Randal J. Kaufman, David Ginsburg, and Uri Seligsohn

Combined factors V and VIII deficiency is an autosomal recessive bleeding disorder associated with plasma levels of coagulation factors V and VIII approximately 5% to 30% of normal. The disease gene was recently identified as the endoplasmic reticulum-Golgi intermediate compartment protein ERGIC-53 by positional cloning, with the detection of two founder mutations in 10 Jewish families. To identify mutations in additional families, the structure of the ERGIC-53 gene was determined by genomic polymerase chain reaction (PCR) and sequence analysis of bacterial artificial chromosome clones containing the ERGIC-53 gene. Nineteen additional families were analyzed by direct sequence

COMBINED DEFICIENCY of coagulation factors V and VIII is an autosomal recessive bleeding disorder with greater than 89 cases reported since the original description by Oeri et al in 1954.¹⁻⁵ Affected individuals present with a moderate bleeding tendency and plasma levels of factors V and VIII (both antigen and activity) in the range of 5% to 30% of normal. Combined factors V and VIII deficiency was initially mapped to the long arm of chromosome 18.⁶⁻⁸ Recent positional cloning efforts identified the disease gene as ERGIC-53, a component of the endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC) of previously unknown function.⁹ This latter report identified two different founder mutations: (1) a G insertion in codon 30 resulting in a frameshift of the 510 amino acid protein product in all affected individuals from five Middle Eastern Jewish families and (2) a splice donor mutation in intron 9 in all affected individuals from five Sephardic Jewish families. Immunofluorescence studies and Western analysis using Epstein-Barr virus (EBV)-immortalized B lymphocytes from affected individuals confirmed the absence of detectable ERGIC-53 antigen.

Although the function of ERGIC-53 is unknown, it is a type 1 transmembrane protein with homology to leguminous lectins that exhibits mannose-selective and calcium-dependent binding and has been hypothesized to play a role in the transport of glycoproteins through the secretory pathway.¹⁰⁻¹⁴ The demonstration that its deficiency results in decreased plasma levels of coagulation factors V and VIII suggests an important role for ERGIC-53 in ER to Golgi transport and implies the existence of cargo-specific pathways within the secretory system. Vollenweider et al¹⁵ have recently shown that mistargeting of ERGIC-53 to the ER impairs the secretion of the lysosomal enzyme cathepsin C, although transport of two other lysosomal enzymes and three post-Golgi membrane glycoproteins was unaffected. These results suggested that the recycling of ERGIC-53 between the ERGIC and ER is required for the intracellular transport of only a small subset of glycoproteins. The common features shared between coagulation factors V and

analysis of the entire coding region and the intron/exon junctions. Seven novel mutations were identified in 10 families, with one additional family found to harbor one of the two previously described mutations. All of the identified mutations would be predicted to result in complete absence of functional ERGIC-53 protein. In 8 of 19 families, no mutation was identified. Genotyping data indicate that at least two of these families are not linked to the ERGIC-53 locus. Taken together, these results suggest that a significant subset of combined factors V and VIII deficiency is due to mutation in one or more additional genes.

© 1999 by The American Society of Hematology.

VIII and other potential ERGIC-53-dependent proteins remain to be determined.

We now report the gene structure for ERGIC-53, as well as mutation analysis for an additional 19 combined factors V and VIII deficiency families. Seven new mutations were identified in 10 of 19 families. In all except one of these families, the affected individual(s) was homozygous for the mutation. One affected individual from a nonconsanguineous family is a compound heterozygote, although the mutation has been identified on only one allele. All of the mutations would predict

From the Division of Human Genetics, Children's Hospital Medical Center, Cincinnati, OH; Howard Hughes Medical Institute and Departments of Medicine, Human Genetics, and Biological Chemistry, University of Michigan, Ann Arbor, MI; Institute of Thrombosis and Haemostasis, The Chaim Sheba Medical Center, Tel-Hashomer and Sackler Faculty of Medicine, Tel Aviv, Israel; the Department of Blood Coagulation and Hemophilia Centre, University Hospital, Policlinico, Bari, Italy; Aichi Blood Disease Research Foundation and The First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya, Japan; Centro Nacional de Hemofilia, Banco Municipal de Sangre, Caracas, Venezuela; the Puget Sound Blood Center and Department of Medicine, University of Washington, Seattle, WA; the Department of Medicine, University of Southern California School of Medicine, Los Angeles, CA; the Department of Medicine, University of North Carolina, Chapel Hill, NC; Edouard Herriot University Hospital, Department of Hematology, Lyon, France; and the Department of Internal Medicine, Istanbul School of Medicine, Istanbul, Turkey.

Submitted September 8, 1998; accepted November 13, 1998.

Supported by Grants No. HL39693 and HL57346 from the National Institutes of Health, Bethesda, MD. D.G. and R.J.K. are investigators of the Howard Hughes Medical Institute.

Address reprint requests to David Ginsburg, MD, 4520 MSRB I, 1150 W Medical Center Dr, Ann Arbor, MI 48109-0650; e-mail: ginsburg@umich.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1999 by The American Society of Hematology.

0006-4971/99/9307-0026\$3.00/0

complete deficiency of a normal ERGIC-53 product. In addition, one of the families was shown to carry the Middle Eastern Jewish founder mutation.⁹ Failure to identify a mutation in eight families, taken together with clear evidence against linkage to the ERGIC-53 locus in two of these families, suggests that a significant subset of combined factors V and VIII deficiency is due to mutations in one or more additional genes.

MATERIALS AND METHODS

ERGIC-53 Gene Structure

Polymerase chain reaction (PCR) and sequence analysis of bacterial artificial chromosomes and total human genomic DNA. Either 2 nanograms of bacterial artificial chromosome (BAC) clones 293H21 or 65B23 (Research Genetics, Huntsville, AL) purified as previously described⁹ or 20 ng of total human genomic DNA was amplified in a 50- μ L reaction containing 200 μ mol/L each dNTP, 50 to 100 ng each primer, 1X PCR buffer (50 mmol/L KCl, 10 mmol/L Tris, pH 9.0, 1.5 mmol/L MgCl₂, 0.01% gelatin, 0.1% Triton X-100), and Taq DNA polymerase. PCR was performed for 35 cycles using an MJ Research PTC-100 96V thermocycler (MJ Research, Watertown, MA). Forward and reverse primers were synthesized from the ERGIC-53 cDNA sequence (GenBank X71661) and used in various combinations to determine the location of introns. PCR products were separated by electrophoresis through agarose and visualized by ethidium bromide staining. Any PCR product whose length was longer than that expected for the cDNA was assumed to contain an intron and was subjected to DNA sequence analysis using the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham Life Science Products, Arlington Heights, IL).

Direct sequence analysis of BACs 293H21 and 65B23. One microgram purified BAC DNA was directly sequenced using the Thermo Sequenase radiolabeled kit as above (Amersham Life Science Products) in a reaction containing the dITP nucleotide master mix and 20 ng of primer specific for the ERGIC-53 coding sequence. Numerous reactions were performed, each with a different specific primer. Cycling conditions were 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 60°C for 6 minutes. Sequencing products were electrophoresed through standard 8% sequencing gels at 100 W. After electrophoresis, the gels were fixed, dried, and exposed to Kodak Biomax film (Eastman Kodak, Rochester, NY).

Mutation analysis in combined factors V and VIII deficiency patients. Twenty-fourty nanograms of genomic DNA, prepared as previously described,⁶ from individuals affected with combined factors V and VIII deficiency (factors V and VIII antigen and activity levels <30% of

normal) and their unaffected parents and siblings (when available) from 19 unrelated families were amplified by PCR. The entire coding region of ERGIC-53 was amplified in multiple PCR reactions using 25 ng (37.5 ng for exon 8) of the appropriate primers as listed in Table 1. Exons 2 and 3 were amplified in a single PCR product as were exons 9 and 10 using the forward primer for exon 2 or 9 and the reverse primer for exon 3 or 10. Only a portion of exon 13 through the stop codon was included in the amplification product. Cycling conditions were 1 minute each of denaturation, annealing, and elongation for 35 cycles using an MJ Research PTC-100 96V thermocycler (MJ Research). PCR products were separated by electrophoresis through 4% agarose (3% Nusieve; FMC Bioproducts, Rockland, ME, 1% BRL) to ensure presence of sufficient quantities for sequence analysis. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Santa Clarita, CA) and eluted in 30 μ L dH₂O. PCR products were sequenced on both strands, as described above, using the same primers as for the PCR reactions.

Haplotype analysis. Haplotype analysis for short tandem repeat markers flanking the ERGIC-53 gene was performed as previously described.⁶ Haplotype analysis using ERGIC-53 intragenic polymorphisms was performed by PCR and sequence analysis as described above.

RESULTS

ERGIC-53 Gene Structure

To determine the intron/exon boundaries of the ERGIC-53 gene, a combination of human genomic and BAC DNA PCR and direct sequence analysis of BAC DNA was used. In previous studies to clone the combined factors V and VIII deficiency gene, BAC clones 293H21 and 65B23, obtained by screening a human genomic DNA BAC library (Research Genetics), had been shown by Southern analysis to contain the largest portions of the ERGIC-53 gene (data not shown) and were therefore used in the PCR and direct sequencing studies.⁹ As seen in Fig 1 and Table 1, the ERGIC-53 gene contains 13 exons ranging in size from 59 bp (exon 7) to greater than 1,244 bp (exon 13). Exon 13 encodes only amino acids 499-510 with the remaining greater than 1,200 bp corresponding to the 3' untranslated sequence (UTR). The full size of the 3' UTR is unknown, as no polyadenylation signal is present in the available cDNA sequence.¹⁶ Exon 1 contains at least 21 bp of 5' UTR, although the transcription start site has not been deter-

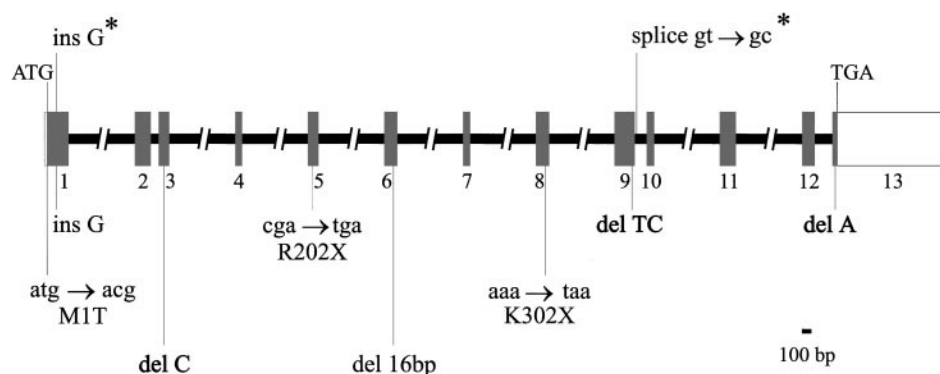


Fig 1. Structure of the ERGIC-53 gene and mutations identified in combined factors V and VIII deficiency. Exons, indicated by rectangles, are numbered from 1 to 13. The coding portion of the gene is shaded, with the open portions of exons 1 and 13 representing the 5' UTR and 3' UTR, respectively. The exons, as well as introns 2, 9, and 12, are shown to scale. The // in the remaining introns indicates that the size was not determined. The locations of the two founder mutations identified in 10 Jewish families⁹ are shown above (indicated by *), while the eight mutations detected by sequence analysis in the 19 families of this study (see Table 2) are shown below the gene.

Table 1. Location and Length of ERGIC-53 Exons and Sequences of Primers Used to PCR Complete Coding Region of ERGIC-53 From Genomic DNA

Exon	Nucleotides*	Exon Length	Primers
1	7-235	235+	5'-TCGCGTTCCAGAATCCAAG-3' 5'-TCAGCACACACAGGGTAGC-3'
2	236-390	155	5'-CAGTTTGGAAATGTACATTGAG-3' 5'-GGGAACAGTTAGAGGCTAG-3'
3	391-498	108	5'-CATGCCTCTAACTGTTCCC-3' 5'-CTCACAGCCTAACTCTGTTG-3'
4	499-560	62	5'-TGTAAGTCACTTCATAGTAC-3' 5'-CAATGTATTTTCATAAGGATTCC-3'
5	561-660	100	5'-TGAAAAGCTGAGTGTCTTGT-3' 5'-GAAAAGTGATACTGTAAACATTG-3'
6	661-784	124	5'-GAAACAAAAGTGAATAGTAGTC-3' 5'-ACAAGTCTACATATCCCTAAv
7	785-843	59	5'-AGAGTGCCATTGCCTTTACC-3' 5'-CAAACCTAAGTTAGTCTTCC-3'
8	844-976	132	5'-CATGTATAGAGATATCTTAATG-3' 5'-GTCCATGATCAACAGCCTC-3'
9	977-1170	194	5'-CACTTTGGTCACTTACGTTA-3' 5'-TCTATGAGCACATAGTACAG-3'
10	1171-1241	71	5'-GGGAAGTAAAGAAGAAGGGC-3' 5'-AATCACATAACACACAACG-3'
11	1242-1395	154	5'-GTGATTTTATTGTATCAAGAG-3' 5'-AGTATGAGTTCTTCTTCC-3'
12	1396-1517	122	5'-GGGGATAGAAGGTTTTCTGG-3' 5'-GAACATAGATAACTTAGTTG-3'
13	1518-?	1244+	5'-CTGTTTCATTTCAAGTTCACAT-3' 5'-AATTCCTCAAACGACATC-3'

*Nucleotide numbers are based on the Genbank file X71661.

mined and the existence of an additional upstream noncoding exon cannot be excluded.

Direct sequence analysis enabled the determination of the size and full sequence for three introns. Introns 2, 9, and 12 were completely sequenced (GenBank Accession numbers AF081867, AF081880, and AF081885) and are 83, 132, 290 bp,

respectively. PCR analysis indicates that intron 1 is approximately 4 kb in length. The sizes of the remaining introns were not determined. All sequence data obtained to date have been deposited in GenBank under accession numbers AF081865, AF081866, AF081867, AF081868, AF081869, AF081870, AF081871, AF081872, AF081873, AF081874, AF081875, AF081876, AF081877, AF081878, AF081879, AF081880, AF081881, AF081882, AF081883, AF081884, and AF081885.

Mutation Analysis in Combined Factors V and VIII Deficiency Patients

We previously reported the identification of two founder mutations in the ERGIC-53 gene in 10 Israeli families with combined factors V and VIII deficiency.⁹ In the current study, we have analyzed DNA samples from 19 additional unrelated families/individuals with the disorder, including 7 families from Italy, 4 from Venezuela, 3 from Japan,¹⁷ 3 from the United States, and 1 each from France and Turkey. The entire coding region of the ERGIC-53 gene and all intron/exon junctions were sequenced in all affected individuals, as well as in selected obligate carrier parents and unaffected siblings of the patients when available (a total of 39 individuals).

Results of the sequence analysis are summarized in Table 2. A total of eight different mutations were identified, accounting for 11 of 19 families. Seven of the eight mutations are either nonsense or frameshift mutations resulting in a truncated protein that would be predicted to lack normal ERGIC-53 function. All but one of the affected individuals is homozygous for their respective mutations, consistent with a high frequency of consanguinity in these families. The affected individual in family 2 is heterozygous for the exon 3 frameshift mutation with no candidate mutation detected on the other allele, either in the ERGIC-53 coding region or the sequences flanking the intron/exon junctions. The mutation detected in family 11, ins G85-89, is the same mutation previously reported in five Middle Eastern Jewish families.⁹ Haplotype analysis of DNA markers in this region demonstrated that the mutation in the affected

Table 2. ERGIC-53 Mutation Analysis in 19 Combined Factors V and VIII Deficiency Families

Family	Country of Origin	ERGIC-53 Gene Mutation*	Amino Acid Substitution
1	Japan	C604 → T, exon 5	Arg202 → stop
2	Japan	del C422, exon 3†	Frameshift at aa 141
3	Japan	Unknown	
4	Italy	T2 → C, exon 1	Met1 → Thr
5	Italy	Unknown	
6	Italy	Unknown	
7	Italy	T2 → C, exon 1	Met1 → Thr
8	Italy	Unknown	
9	Italy	T2 → C, exon 1	Met1 → Thr
10	Italy	T2 → C, exon 1	Met1 → Thr
11	Venezuela (Iranian Jewish)	ins G85-89, exon 1	Frameshift at aa 31
12	Venezuela	del A720-A735 (16 bp), exon 6	Frameshift at aa 240
13	Venezuela	Unknown	
14	Venezuela	Unknown	
15	France	A904 → T, exon 8	Lys302 → stop
16	Turkey	Unknown	
17	United States (Armenian)	del A1519-1524, exon 13	Frameshift at aa 508
18	United States	del TC1109-1112, exon 9	Frameshift at aa 371
19	United States	Unknown	

*Nucleotide numbers are based on the Genbank file X71661 using the A (nucleotide 22) of the ATG initiator methionine as +1.

†Patient is heterozygous for this mutation with no mutation detected on the other allele.

Table 3. Polymorphisms of the ERGIC-53 Gene

Location	Polymorphism
Exon 1-A	G31 → A, Arg14 → Gln
Exon 1-B	C116 → T, Val39 → Arg
Exon 2	G351 → A, silent
Intron 4	del GT(+5 → +12)
Intron 6*	ins T in pyrimidine tract T ₁₂ at 3' of intron (-10 → -21)
Intron 7*	ins TGGT(+40)
Exon 8	C936 → T, silent
Exon 11	A1228 → T, Met410 → Leu
Intron 11	C(+14) → T

*These two intron polymorphisms are in complete linkage disequilibrium.

individual in this family, also of Middle Eastern Jewish origin (Iranian-Jewish) is carried on the same haplotype,⁶ consistent with a common origin (data not shown).

A single, common mutation was identified in four of the seven Italian families in our patient cohort (Table 2). The affected individuals in these four families were homozygous for the only missense mutation observed in our study. This results in the substitution of threonine for the initiator methionine. The next methionine codon is not encountered until exon 2 and is out of frame. This mutation should thus result in the complete absence of ERGIC-53 expression in these patients. In all four of the families, the mutation is carried on a shared haplotype, again suggesting a founder effect.

A number of additional sequence differences also detected in the ERGIC-53 gene were identified as likely polymorphisms or rare sequence variants (Table 3). Most of these changes were observed in numerous unrelated families and are likely to represent common polymorphisms. The intron 6 polymorphism appears to be in complete linkage disequilibrium with the intron 7 polymorphism; that is, the two polymorphisms are always inherited together with no individual identified to carry only one.

Of note, all chromosomes sequenced to date contain a

thymine at nucleotide 457, not the adenine reported in the published cDNA¹⁶, resulting in a serine residue at position 153 in place of the reported threonine. An independently reported sequence for a mannose-specific lectin termed MR60¹² is identical to ERGIC-53 except for the same serine for threonine substitution at position 153. This suggests a sequencing or cloning artifact in the original report, although a rare sequence variant in the source of the cDNA library cannot be excluded.

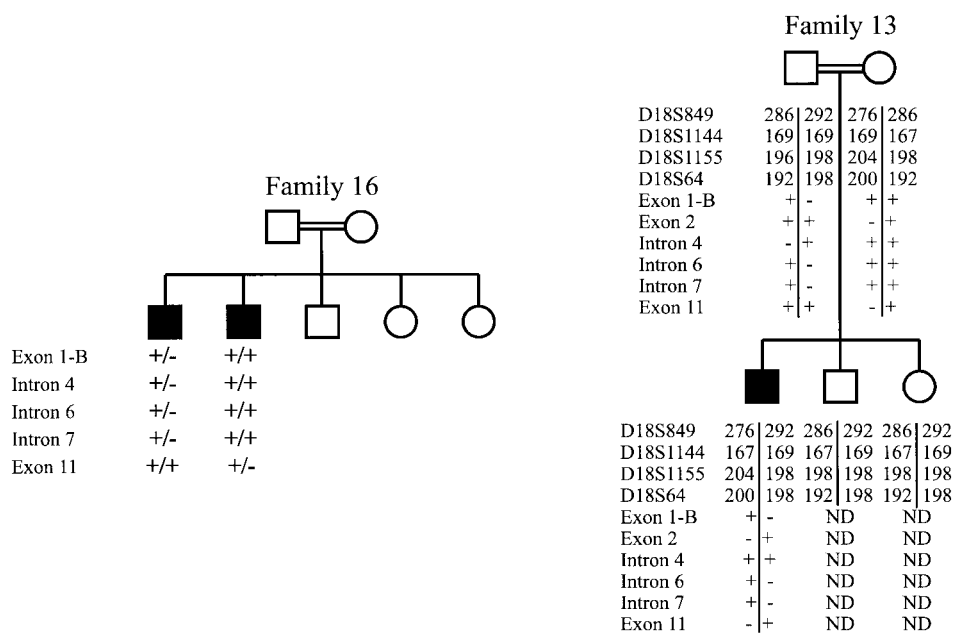
Haplotype Analysis in ERGIC-53 Mutation-Negative Families

Shown in Fig 2 is haplotype analysis for two of the eight families in our study for which no mutation was detected in the entire ERGIC-53 coding sequence or intron sequences flanking the intron/exon junctions. The affected siblings in family 16 inherited different ERGIC-53 alleles from their parents, indicating lack of linkage to the ERGIC-53 gene. The parents of the affected individual in family 13 are first cousins, making it likely that the patient is homozygous for the same mutant allele inherited from the carrier parents. However, the patient is heterozygous for four short tandem repeat markers flanking the ERGIC-53 gene, as well as five of six intragenic polymorphisms. These results indicate that the mutations responsible for combined factors V and VIII deficiency in these two families are unlikely to lie within or near the ERGIC-53 gene.

DISCUSSION

We report the identification of seven novel mutations in the ERGIC-53 gene in patients with combined factors V and VIII deficiency. Mutations were identified in 11 of 19 families studied. One founder mutation, by virtue of haplotype analysis, was identified in four of seven Italian families. All patients, except one, are homozygous for the respective mutation, consistent with the high frequency of consanguinity in these families. The large number of families reported here and in the accompanying report¹⁸ suggests that combined factors V and VIII deficiency may be considerably more frequent than

Fig 2. Haplotype analysis for two families in which no ERGIC-53 mutation was detected. Affected individuals (shaded) in family 16, shown at the left, were determined to differ at five ERGIC-53 intragenic polymorphisms (Table 3). The + indicates the presence of the more common sequence, while the - indicates the presence of the polymorphism or rare sequence variant. Individuals in family 13, shown at the right, were genotyped for four short tandem repeat polymorphisms flanking the ERGIC-53 gene.^{6,9} Numbers represent basepair length of the PCR products generated with primers flanking the repeat sequence. Both parents and the affected individuals were also typed for six intragenic polymorphisms (Table 3).



originally suspected. However, consistent with previous reports,²⁻⁵ the majority of families in both studies are of Mediterranean/Middle Eastern origin. Taken together with the evidence for a founder effect among Middle Eastern Jews⁹ (and this study), Sephardic Jews,⁹ Italians (this study), Iranians,¹⁸ and Pakistanis,¹⁸ these data suggest the possibility of a specific positive selection for heterozygotes among individuals in these geographic areas, perhaps resistance to a regional infectious pathogen, as has been elegantly demonstrated for the hemoglobinopathies.¹⁹

ERGIC-53 is a type 1 transmembrane protein that constitutively recycles between the ER, the ERGIC, and *cis* Golgi.^{10,14,16} The recent identification of ERGIC-53 as the gene responsible for combined factors V and VIII deficiency provided the first direct evidence that this protein might function as a sorting receptor in the ER for a specific subset of secretory proteins.⁹ The C-terminal sequence of ERGIC-53 (507LysLysPhePhe510) contains a dilysine ER retrieval signal.²⁰ Kappeler et al²¹ have demonstrated by site directed mutagenesis that the two terminal phenylalanine residues are required for exit of ERGIC-53 from the ER. These signals also cooperate with luminal and transmembrane domains in the normal intracellular trafficking of ERGIC-53. Vollenweider et al¹⁵ recently studied a recombinant mutant ERGIC-53 in which AlaAla had replaced the diphenylalanine motif. This mutant was prevented from recycling and accumulated in the ER, exerting a dominant-negative effect on the endogenous normal ERGIC-53. A selective delay in secretion of the lysosomal protein cathepsin C was observed, consistent with the proposed role of ERGIC-53 as a specific sorting receptor. The absence of any other apparent disease phenotype in combined factors V and VIII deficiency patients, outside of the bleeding disorder, suggests that the defect in the targeting of cathepsin C or other similar proteins may be too subtle to produce a significant functional abnormality.

All eight mutations reported here result in premature termination or truncation removing the LysLysPhePhe motif. The most distal of these mutations, a one base deletion in exon 13, results in a frameshift at amino acid 508 (Table 2, family 17). This mutation substitutes LysAsnSerPhe for the LysLysPhePhe motif, followed by 14 additional amino acids before the next stop codon. Despite the presence of greater than 99% (507 of 510 amino acids) of the ERGIC-53 protein, the predicted protein, if stably folded and able to exit the ER, lacks the critical dilysine ER retention signal²⁰ and would likely be constitutively secreted from the cell. In addition to deleting the KKFF motif, all of the other identified mutations also remove the transmembrane domain and cytoplasmic domain.²²

Only one missense mutation has been identified to date in the ERGIC-53 gene in patients with combined factors V and VIII deficiency. Detected in 4 of 7 Italian families included in the study, this single nucleotide change results in the substitution of the initiator methionine with threonine. As the next methionine codon is not encountered until exon 2 and it is out of frame, no functional ERGIC-53 would be predicted in these patients. This same mutation was identified by Neerman-Arbez et al¹⁸ in 2 of 8 Italian families in their study of 35 families. The finding of only mutations that are completely inactivating suggests that even minimal residual ERGIC-53 function might be sufficient for normal factor V and factor VIII secretion.

Although all 10 Middle Eastern and Sephardic Jewish families in our original report had an identifiable mutation, this result was biased by the finding of two founder mutations accounting for all patients in this restricted population.⁹ In contrast, no ERGIC-53 mutation was detected in 8 of 19 families in the current study. Taken together with our previous study of 10 Israeli families, we have failed to find a mutation in approximately 30% (8 of 29) of the families studied. These latter results are similar to those of Neerman-Arbez et al,¹⁸ who were unable to detect a mutation in nine of 35 families. Mutations in portions of the ERGIC-53 gene that were not sequenced, such as upstream regulatory regions, 3' UTR, or intron sequences could account for these patients, although mutations of this type are generally infrequent in other genetic disorders. However, a hot spot for recurrent mutation, as results in the common FVIII gene inversion in hemophilia A, could account for this observation.²³ The affected individual in family two, for whom the mutation on only one allele was identified, is most likely explained as a compound heterozygote with an undetected mutation in the other allele of ERGIC-53. This patient's plasma levels of factors V and VIII (12% FV, 14% FVIII)¹⁷ are similar to those of other combined factors V and VIII deficiency patients, offering no particular insight into the nature of the second, undetected mutation.

Our data for lack of linkage to ERGIC-53 in two mutation-negative families (Fig 2) provide compelling evidence that the combined factors V and VIII deficiency in at least a subset of these patients is due to mutations in another gene. Consistent with this hypothesis, Neerman-Arbez et al¹⁸ observed apparently normal levels of ERGIC-53 by Western analysis of cell extracts from EBV-immortalized B lymphocytes in two of three families for which no ERGIC-53 mutation was detected by single-strand conformation polymorphism and sequence analysis. Taken together with our linkage findings, these data strongly suggest the presence of at least one additional locus for combined factors V and VIII deficiency. No phenotypic differences are discernible between affected individuals for whom an ERGIC-53 mutation has been detected and mutation-negative families or the specific subset demonstrating lack of linkage to the ERGIC-53 locus. It is likely that the gene(s) encoded by this alternative locus also functions in the secretory pathway for coagulation factors V and VIII, perhaps acting directly upstream or downstream of ERGIC-53. The future identification of this alternative gene(s) is likely to shed important light on the biological function of this unique cargo-specific ER to Golgi transport pathway.

ACKNOWLEDGMENT

We are indebted to the families who donated samples for this study and to Dr Tadashi Kamiya for assistance with the Japanese families.

REFERENCES

1. Oeri J, Matter M, Isenschmid H, Hauser F, Koller F: Angeborener mangel an faktor V (parahaemophilie) verbunden mit echter haemophilie A bei zwei brudern. *Med Probl Paediatr* 1:575, 1954
2. Seligsohn U: Combined factor V and factor VIII deficiency, in Seghatchian J, Savidge GT (eds): *Factor VIII-Von Willebrand Factor*. Boca Raton, FL, CRC, 1989, p 89
3. Seligsohn U, Ramot B: Combined factor-V and factor-VIII deficiency: Report of four cases. *Br J Haematol* 16:475, 1969

4. Seligsohn U, Zivelin A, Zwang E: Combined factor V and factor VIII deficiency among non-ashkenazi Jews. *N Engl J Med* 307:1191, 1982
5. Seligsohn U, Zivelin A, Zwang E: Decreased factor VIII clotting antigen levels in the combined factor V and VIII deficiency. *Thromb Res* 33:95, 1984
6. Nichols WC, Seligsohn U, Zivelin A, Terry VH, Arnold ND, Siemieniak DR, Kaufman RJ, Ginsburg D: Linkage of combined factors V and VIII deficiency to chromosome 18q by homozygosity mapping. *J Clin Invest* 99:596, 1997
7. Neerman-Arbez M, Antonarakis SE, Blouin J-L, Akhtari M, Afshar Y, Tuddenham EGD: The locus for combined factor V-factor VIII deficiency (F5F8D) maps to 18q21, between D18S849 and D18S1103. *Am J Hum Genet* 61:143, 1997
8. Ginsburg D, Nichols WC, Kaufman RJ, Seligsohn U: Combined factors V and VIII deficiency — the solution. *Haemophilia* 4:677, 1998
9. Nichols WC, Seligsohn U, Zivelin A, Terry VH, Hertel CE, Wheatley MA, Moussalli MJ, Hauri H-P, Ciavarella N, Kaufman RJ, Ginsburg D: Mutations in the ER-Golgi intermediate compartment protein ERGIC-53 cause combined deficiency of coagulation factors V and VIII. *Cell* 93:61, 1998
10. Schweizer A, Fransen JAM, Bachi T, Ginsel L, Hauri H-P: Identification, by a monoclonal antibody, of a 53-kD protein associated with a tubulo-vesicular compartment at the *cis*-side of the Golgi apparatus. *J Cell Biol* 107:1643, 1988
11. Itin C, Roche A-C, Monsigny M, Hauri H-P: ERGIC-53 is a functional mannose-selective and calcium-dependent human homologue of leguminous lectins. *Mol Biol Cell* 7:483, 1996
12. Arar C, Carpentier V, Le Caer JP, Monsigny M, Legrand A, Roche AC: ERGIC-53, a membrane protein of the endoplasmic reticulum-Golgi intermediate compartment, is identical to MR60, an intracellular mannose-specific lectin of myelomonocytic cells. *J Biol Chem* 270:3551, 1995
13. Fiedler K, Simons K: A putative novel class of animal lectins in the secretory pathway homologous to leguminous lectins. *Cell* 77:625, 1994
14. Schweizer A, Fransen JA, Matter K, Kreis TE, Ginsel L, Hauri HP: Identification of an intermediate compartment involved in protein transport from endoplasmic reticulum to Golgi apparatus. *Eur J Cell Biol* 53:185, 1990
15. Vollenweider F, Kappeler F, Itin C, Hauri H-P: Mistargeting of the lectin ERGIC-53 to the endoplasmic reticulum of HeLa cells impairs the secretion of a lysosomal enzyme. *J Cell Biol* 142:377, 1998
16. Schindler R, Itin C, Zerial M, Lottspeich F, Hauri H-P: ERGIC-53, a membrane protein of the ER-Golgi intermediate compartment, carries an ER retention motif. *Eur J Cell Biol* 61:1, 1993
17. Suzuki K, Nishioka J, Hashimoto S, Kamiya T, Saito H: Normal titer of functional and immunoreactive protein-C inhibitor in plasma of patients with congenital combined deficiency of factor V and factor VIII. *Blood* 62:1266, 1983
18. Neerman-Arbez M, Johnson KM, Morris MA, McVey JH, Peyvandi F, Nichols WC, Ginsburg D, Rossier C, Antonarakis SE, Tuddenham EGD: Molecular analysis of the ERGIC-53 gene in 35 families with combined factor V-factor VIII deficiency (F5F8D). *Blood* 93:2253, 1999
19. Kazazian HH, Jr: The thalassemia syndromes: Molecular basis and prenatal diagnosis in 1990. *Semin Hematol* 27:209, 1996
20. Jackson MR, Nilsson T, Peterson PA: Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO J* 9:3153, 1990
21. Kappeler F, Klopfenstein DRCh, Foguet M, Paccaud J-P, Hauri H-P: The recycling of ERGIC-53 in the early secretory pathway. *J Biol Chem* 272:31801, 1997
22. Itin C, Schindler R, Hauri H-P: Targeting of protein ERGIC-53 to the ER/ERGIC/*cis*-Golgi recycling pathway. *J Cell Biol* 131:57, 1995
23. Lakich D, Kazazian HH Jr, Antonarakis SE, Gitschier J: Inversions disrupting the factor VIII gene are a common cause of severe haemophilia A. *Nat Genet* 5:236, 1993