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Mutations in the ER–Golgi Intermediate Compartment Protein ERGIC-53 Cause Combined Deficiency of Coagulation Factors V and VIII

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Summary

Combined deficiency of factors V and VIII is an autosomal recessive bleeding disorder resulting from alterations in an unknown gene on chromosome 18q, distinct from the factor V and factor VIII genes. ERGIC-53, a component of the ER–Golgi intermediate compartment, was mapped to a YAC and BAC contig containing the critical region for the combined factors V and VIII deficiency gene. DNA sequence analysis identified two different mutations, accounting for all affected individuals in nine families studied. Immunofluorescence and Western analysis of immortalized lymphocytes from patients homozygous for either of the two mutations demonstrate complete lack of expression of the mutated gene in these cells. These findings suggest that ERGIC-53 may function as a molecular chaperone for the transport from ER to Golgi of a specific subset of secreted proteins, including coagulation factors V and VIII.

Introduction

Coagulation factor V (FV) and factor VIII (FVIII) are both required for the efficient function of the blood-clotting system. Genetic deficiency of FVIII results in classic hemophilia (hemophilia A), an X chromosome–linked bleeding disorder afflicting ~1 in 5000 males. Inherited FV deficiency, or parahemophilia, is a rare autosomal recessive condition exhibiting a similar hemorrhagic phenotype (Ginsburg, 1997). Combined deficiency of both factors V and VIII is a distinct clinical entity first described by Oeri et al. (1954). Affected patients demonstrate a moderate bleeding tendency in association with plasma levels of FV and FVIII (both antigen and activity) in the range of 5%–30% of normal. Since this original report, more than 89 patients belonging to at least 58 families have been described. Of the 58 families, 24 are of Mediterranean origin including 9 Italian and 9 Israeli families. The remaining 34 families are from North America, Europe, and Japan (Seligsohn, 1989). This disorder appears to be particularly prevalent among Jews of Middle Eastern and Sephardic origin living in Israel with an estimated frequency of 1:100,000 (Seligsohn et al., 1982). Inheritance is autosomal recessive and is distinct from coinheritance of both FV deficiency (parahemophilia) and FVIII deficiency (hemophilia A) (Girolami et al., 1976; Mazzone et al., 1982; Ozsoylu, 1983; Seligsohn, 1989). Affected individuals are assumed to be homozygous at a locus, which, in some manner, uniquely regulates the expression of these two specific proteins.

FV is synthesized primarily in megakaryocytes and hepatocytes and is found in the plasma and α granules of platelets as a 330 kDa single chain polypeptide (Chiu et al., 1985). FVIII is most likely synthesized in the hepatocyte and reticuloendothelial cells (Wion et al., 1985; Bontempo et al., 1987) and is processed upon secretion from the cell to a heterodimer consisting of a carboxy-terminal–derived light chain of 80 kDa in a metal ion–dependent association with a 200 kDa amino-terminal–derived heavy chain fragment. FV and FVIII circulate in plasma as inactive precursors that are activated through limited proteolysis and subsequently assemble with their respective substrates (prothrombin and factor X) and enzymes (factor Xa and factor IXa) on a negatively charged phospholipid surface. FVIII stability in plasma requires a tight noncovalent interaction with the plasma protein von Willebrand factor (vWF) (for review, see (Kaufman, 1998). Plasma FV does not require a similar carrier protein, and its concentration exceeds that of FVIII by approximately 40-fold.

FV and FVIII are homologous proteins that have a conserved domain organization. The A domains of FVIII share 40% amino acid identity with each other and to the A domains of FV and also to the copper binding protein ceruloplasmin (Ortel et al., 1984). Both FV and FVIII contain 1 mol copper ion/mol protein, and at least for FVIII, this copper ion is in the reduced form (Mann et al., 1984; Tagliavacca et al., 1997). The FVIII C domains also exhibit 40% identity to each other, and to the C domains of FV, and share homology with proteins that bind negatively charged phospholipids (Stubbs et al., 1990). The B domains demonstrate little homology at the amino acid sequence level but are encoded by single exons of similar length and are both heavily glycosylated (Gitschier et al., 1984; Cripe et al., 1992). FV and FVIII undergo similar and extensive posttranslational modifications that include formation and conserved pairing of disulfide bonds (Kaufman, 1998), addition and complex modification of multiple asparagine-linked oligosaccharide residues (Pittman et al., 1994b), addition of multiple

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serine/threonine linked oligosaccharides within the B domain, and sulfation of multiple tyrosine residues (Michnick et al., 1994; Pittman et al., 1994c).

The molecular basis for combined factors V and VIII deficiency has been a puzzle since its first description in 1954 (Oeri et al., 1954). Chance coinheritance of classic hemophilia A (FVIII deficiency) and parahemophilia (FV deficiency) has been reported in four families (Girolami et al., 1976; Mazzone et al., 1982; Ozsoylu, 1983). However, this explanation cannot account for the majority of combined deficiency patients, for whom the genetic pattern reflects simple autosomal recessive inheritance due to a defect in a single gene. FV and FVIII are both proteolytically inactivated by activated protein C (APC). Thus, deficiency of protein C inhibitor, leading to unopposed increased activity of APC, seemed a logical explanation for this disorder when first reported (Marlar and Griffin, 1980). However, subsequent studies documented normal protein C inhibitor levels in these patients and demonstrated that the initial observation of decreased levels was due to a laboratory artifact (Canfield and Kisiel, 1982; Suzuki et al., 1983; Gardiner and Griffin, 1984; Rahim Adam et al., 1985). The normal survival of exogenous coagulation factors when administered to combined deficiency patients argues against an accelerated clearance mechanism and suggests that this disorder might be due to a common defect in biosynthesis or secretion (Seligsohn, 1989). However, this latter explanation is difficult to reconcile with the markedly different plasma levels and patterns of expression for these two proteins.

We recently localized the gene for combined factors V and VIII deficiency to the long arm of chromosome 18 in nine unrelated Jewish families of Sephardic and Middle Eastern origin using a homozygosity mapping approach (Nichols et al., 1997). This localization was subsequently confirmed in an independent study of 19 families from Iran, Pakistan, and Algeria (Neerman-Arbez et al., 1997). We now report identification of the gene responsible for combined factors V and VIII deficiency and the characterization of two distinct founder mutations. The combined factors V and VIII deficiency gene corresponds to a well-described marker of the endoplasmic reticulum–Golgi intermediate compartment, ERGIC-53 (Schweizer et al., 1988, 1990). ERGIC-53, a type 1 transmembrane protein with homology to leguminous lectins (Fiedler and Simons, 1994), exhibits mannose-selective and calcium-dependent binding (Arar et al., 1995; Itin et al., 1996) and has recently been hypothesized to play a role in the transport of glycoproteins through the secretory pathway.

Results

Construction of Physical Map for Genomic Region Containing the Combined Factors V and VIII Deficiency Gene

In order to identify the gene responsible for combined factors V and VIII deficiency, it was first necessary to construct a physical map of the region (Figure 1). Previous genetic linkage studies and recombination analysis had localized the gene to a 2.5 cM region between the genetic markers D18S1144 and D18S1109 (Nichols et al., 1997). YAC clones spanning the 3.2 cM genetic interval from D18S849 to D18S64 were identified from the Human Physical Mapping Project at the Whitehead Institute for Biomedical Research/MIT Center for Genome Research. PCR was used to confirm that the YAC clones obtained were positive for the appropriate markers, as reported. PFGE and Southern analysis determined the size of the YACs and enabled determination of the physical distance between markers spanning the critical interval. A complete set of overlapping YACs was established with an estimated size of 3.0 megabases (Mb) between D18S849 and D18S64 (Figure 1), consistent with the genetic distance of 3.2 cM determined previously using Multimap. A partial BAC contig was also constructed by screening a human BAC library with the seven genetic markers, four STSs, and 11 ESTs (see Figure 1) that were used to construct the YAC contig. Thirty-three clones were identified spanning the interval from D18S1144 to D18S64. A complete overlapping set of BAC clones was established for the interval D18S1103–D18S64. End clone sequences were determined for ten of the clones by direct sequence analysis of whole BAC DNA. STSs were developed from the end clone sequences and used to determine overlap between the clones. NotI digestion followed by PFGE was used to determine the insert size for a number of the BAC clones in the contig (Figure 1).

Identification of Candidate Genes for Combined Factors V and VIII Deficiency

To begin the characterization of potential candidate genes lying within the YAC contig, 36 ESTs that mapped in radiation hybrid bins which would contain the entire candidate interval of D18S1144–D18S1109 were identified from the human transcript map (Schuler et al., 1996). Because the liver appears to be a primary site of synthesis for both FV and FVIII (Chiu et al., 1985; Wion et al., 1985), we hypothesized that the combined factors V and VIII deficiency gene should also be expressed in that tissue. Therefore, we focused our initial analysis on those ESTs that had been isolated, at least in part, from liver mRNA. Of the 36 ESTs/genes, 19 fulfilled this criteria and were considered to be potential candidate genes (Figure 1). The YAC and BAC clones comprising the critical region contig were analyzed by PCR for each of the 19 ESTs/genes. Of the 19 expressed sequences, 11 were shown to map to the candidate interval (Figure 1). Linkage analysis of 19 additional families by Neerman-Arbez et al. (1997) confirmed our localization of the combined factors V and VIII deficiency gene to chromosome 18q and placed the gene between D18S849 and D18S1103. In contrast, our analysis of an additional affected individual from an Italian family identified a recombination between D18S1103 and D18S1155 (data not shown) placing the gene between D18S1103 and D18S1109 (Figure 1) and excluding the segment from D18S849 to D18S1103 suggested by Neermaj-Arbez et al. (1997). The further narrowing of the candidate interval based on our additional patient resulted in the elimination of 8 of the 11 ESTs as candidate genes.

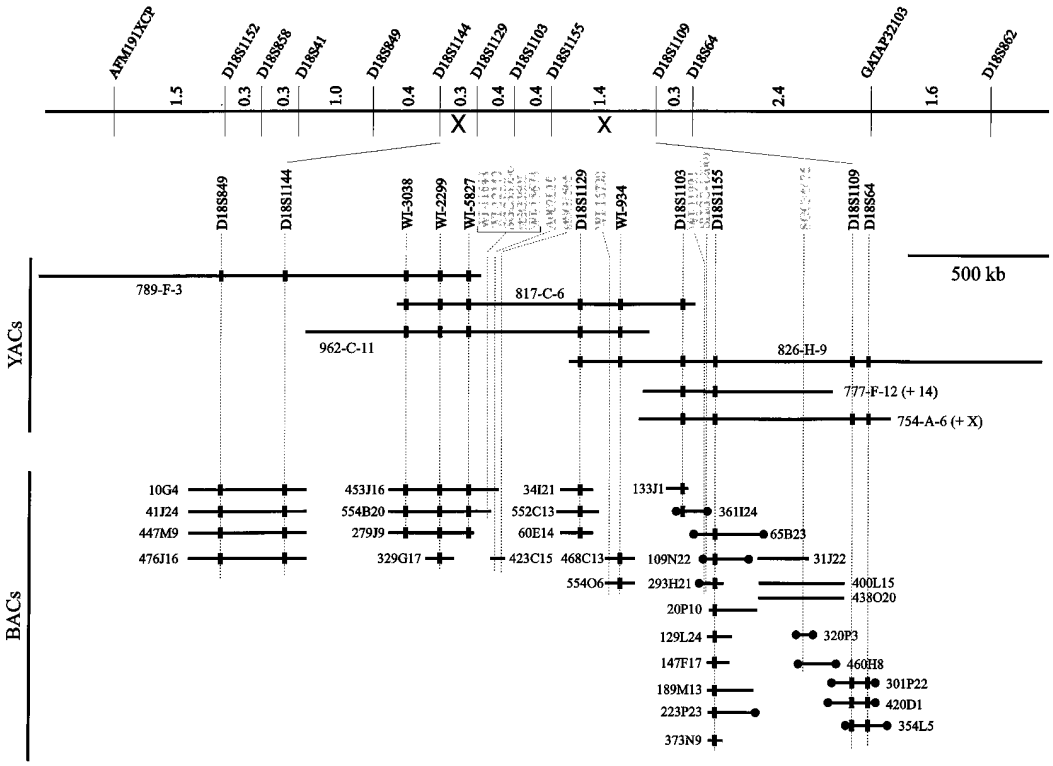

Figure 1. Physical Mapping of Critical Interval

At the top is shown the genetic linkage map for the 13 polymorphic markers used in the original linkage analysis (Nichols et al., 1997). The X's indicate the critical recombinants placing the combined factors V and VIII deficiency gene in the 2.5 cM region between markers D18S1144 and D18S1109. The region between markers D18S849 and D18S64 is enlarged below. The location of individual polymorphic markers and STSs (in black) and ESTs (in gray) are indicated. Complete YAC and partial BAC contigs were assembled as described in the text, with the approximate scale indicated. YAC clones were sized by pulsed-field gel electrophoresis. YAC sizes were not determined for clones 777-F-12 and 754-A-6, which are reportedly chimeric with chromosome 14 and X, respectively. All BAC clones spanning the interval from D18S1103 to D18S64 (except 31J22, 400L15, and 328O20) were sized by pulsed-field gel electrophoresis. Dashed lines extending downward from the polymorphic markers; STSs and ESTs at the top of the physical map indicate YAC clones and BAC clones that were positive for each marker. Closed circles at the end of some BAC clones indicate end clone sequences.

Mutation Analysis for *ERGIC-53*

One of the ESTs that mapped to the recombinant interval (SHGC-12001, accession number X71661) corresponded to the cDNA for ERGIC-53, a previously described resident protein of the endoplasmic reticulum–Golgi intermediate compartment of unknown function (Schweizer et al., 1988, 1990; Schindler et al., 1993; Itin et al., 1996). The complete coding sequence for *ERGIC-53* was amplified by PCR in three overlapping fragments (Table 1) using total RNA isolated from EBV-transformed B-lymphocyte cultures established from two of the Middle Eastern Jewish patients (2-1 and 3-1 in Figure 1 of Nichols et al., 1997) and one of the Sephardic Jewish patients (6-3) with combined factors V and VIII deficiency and one normal individual. DNA sequence analysis was performed on both strands for the entire coding region. Homozygosity for a single base insertion of guanine in a stretch of four guanines from bases 86–89 (A of ATG initiatior = +1) was identified in both Middle Eastern Jewish patients (See Figure 2). The single base insertion predicts a frameshift at codon 30 resulting in a truncated protein containing only the first 30 N-terminal amino acids of ERGIC-53 followed by 71 residues in the new reading frame leading up to the first stop codon.

RNA PCR of fragment 2 (Table 1) yielded an aberrant PCR product in the Sephardic Jewish patient 6–3. This product is approximately 150 base pairs larger than that observed from the RNAs of two control individuals as well as the two Middle Eastern Jewish patients (2-1 and 3-1) (Figure 3). DNA sequence analysis of this fragment identified homozygosity for a 132 base pair insertion in the Sephardic Jewish patient with GC at the 5′ end and AG at the 3′ end of the insertion as compared to the normals and Middle Eastern Jewish patients (Figure 3). Amplification of the corresponding region from genomic DNA generated a 346 base pair fragment (214 base pairs of coding + 132 base pairs of intron, see Table 1) in all samples. The intron sequence corresponds to the 132 bp insertion amplified from the mRNA of patient 6-3. DNA sequence analysis of genomic DNA PCR product confirmed homozygosity for the thymine-to-cytosine change at the consensus splice donor in the Sephardic Jewish patient (Figure 3). No evidence of the correctly spliced mRNA product is detected in patient 6-3 (Figure 3), indicating that this mutation results in complete loss of splicing of this intron. This mutation would predict a protein product that included the N-terminal 383 (of 510)

Table 1. **ERGIC-53** PCR Product Primers and Allele-Specific Oligonucleotides

	Nucleotides[a]	Primer Sequences	Product Length (bp)	Annealing Temp (°C)
PCR product				
RNA PCR fragment 1	−19–810	TCGCGTTCCAGAATCCAAG (F)[b] AGGTTCAGTCAACTGGAAAG (R)	829	56
RNA PCR fragment 2	711–1593	TATCCCTGCACAAGGGCATT (F) AATTCCCTCAAAACGACATC (R)	883	54
RNA PCR fragment 3	1484–2079	ATATCATGTATAGGTCTCAG (F) TTGAAGTCTATTACCAGATC (R)	596	52
RNA PCR to detect splice mutation	990–1299	GCTAAGACAAGTCTTTGCAG (F) TGAAGTGCTGTGTTGTCTCA (R)	320	54
DNA PCR to detect splice mutation	990–1203	GCTAAGACAAGTCTTTGCAG (F) TCTCAGAATCTCATGCTGAG (R)	214 + 132 (intron)	55
DNA PCR to detect G insertion mutation	50–139	TCTGCGCCTTGCTGCTGTCA (F) CGAAACGGCGATGTGGCAAC (R)	90	62
Allele-Specific oligonucleotides (ASO)				
ASO to detect G insertion mutation	80–93 (+G)	TCGTCCGGGGGCGAC		
ASO to detect normal sequence	80–94	TCGTCCGGGGCGACG		
ASO to detect T-to-C splice donor mutation	1144–1149 + 1–9 (intron)	GGGCAGGCGGGCTTG		
ASO to detect normal splice donor	1144–1149 + 1–9 (intron)	GGGCAGGTGGGCTTG		

[a] Based on **ERGIC-53** cDNA (GDB accession number X71661) sequence with A of initiatior ATG = +1 (nucleotide 21 of X71661).
[b] (F) and (R) indicate forward (sense) and reverse (antisense) primers.

amino acids of ERGIC-53 followed by 18 residues encoded by the unspliced intron leading up to the first in-frame stop codon.

Our previous genetic linkage analysis of nine Sephardic Jewish and Middle Eastern Jewish families identified two distinct haplotypes segregating with the disease, suggesting the possibility of two independent founder chromosomes. The five Sephardic Jewish families shared a haplotype extending for at least 6.5 cM including the markers D18S1103–D18S862 (Figure 1) while the four Middle Eastern Jewish families shared a different haplotype covering a 0.4–2.2 cM region containing only the markers D18S1103 and D18S1155. These data suggested that the origin of the mutation in the Middle Eastern Jewish families may be more ancient than that in the Sephardic Jewish families (Nichols et al., 1997).

DNA samples from members of all nine families were screened by hybridization with allele-specific oligonucleotide probes specific for the two mutations identified above (Table 1). All 9 affected individuals from the 4 Middle Eastern Jewish families were homozygous for the *ERGIC-53* codon 30 G insertion mutation while all 6 affected individuals from the 5 Sephardic Jewish families were homozygous for the splice donor mutation (Figure 4). In all cases, parents of the affected individuals were heterozygous carriers of the corresponding mutations. All individuals homozygous for either mutation were clinically affected with combined factors V and VIII deficiency, confirming 100% penetrance for this disorder. Analysis of an additional Middle Eastern Jewish family originally from Iran, not included in our original mapping report (Nichols et al., 1997), again identified

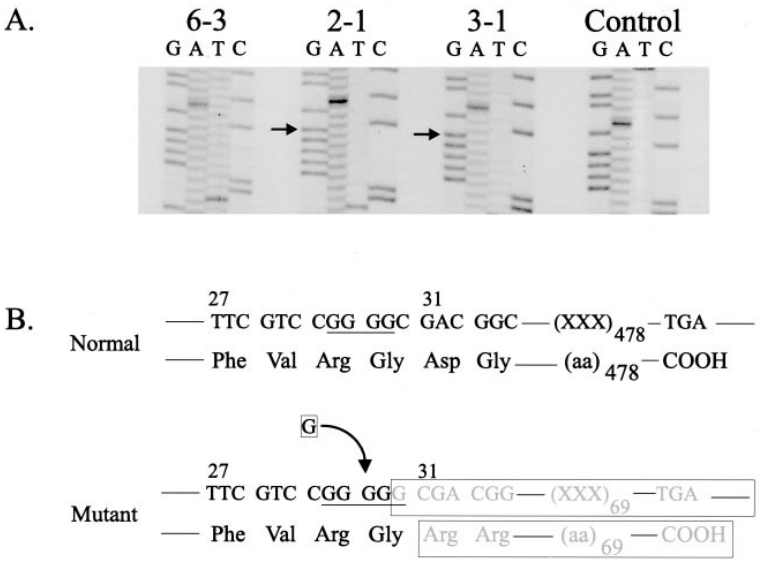

Figure 2. Frameshift Mutation in *ERGIC-53*

(A) Sequence analysis of RT-PCR products of fragment 1 of the *ERGIC-53* coding sequence (Table 1) derived from a Sephardic Jewish combined factors V and VIII deficiency patient (6-3), two Middle Eastern Jewish patients (2-1 and 3-1), and a control individual. Numbers from individual patients correspond to those used in the pedigrees shown in Figure 1 of Nichols et al. (1997). The Middle Eastern Jewish patients are both homozygous for a G insertion in a run of 4 G's corresponding to base pairs 86–89 of the *ERGIC-53* cDNA. (B) The single base insertion predicts a frameshift at codon 30 resulting in a truncated protein containing only the first 30 N-terminal amino acids of ERGIC-53 followed by 71 residues in the new reading frame leading up to the first stop codon.

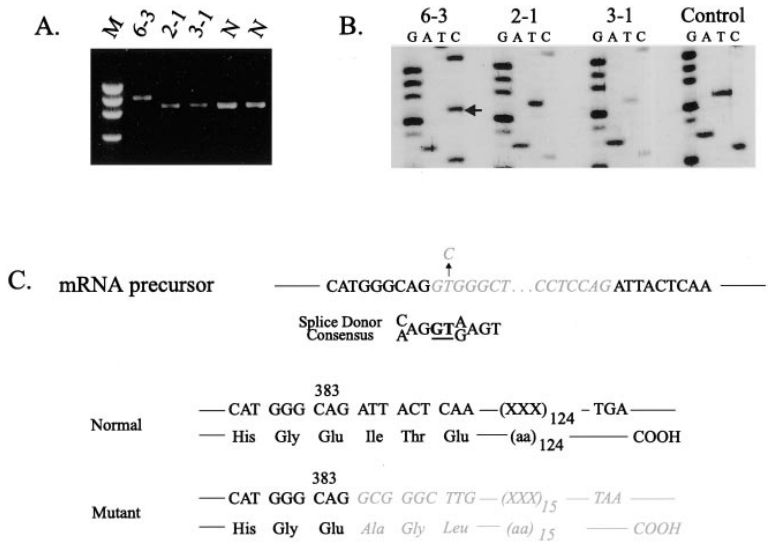

Figure 3. Splice Donor Mutation in ERGIC-53

(A) Ethidium bromide–stained agarose gel of RT-PCR product for *ERGIC-53* "RNA PCR fragment 2" (Table 1) amplified from RNA derived from combined factors V and VIII deficiency patients 6-3, 2-1, and 3-1 (see legend to Figure 2). The marker (M) is HaeIII digest of FX174.

(B) DNA sequence analysis of PCR products for the corresponding region of genomic DNA ("DNA PCR to detect splice mutation," Table 1). Patient 6-3 is homozygous for a thymine-to-cytosine substitution in the highly conserved splice donor consensus. Analysis of the RT-PCR product in (A) is consistent with complete failure to remove this intron in patient 6-3.

(C) The predicted translation product from the mutant allele contains the N-terminal 383 (of 510) amino acids of ERGIC-53 followed by 18 residues encoded by the unspliced intron leading up to the first in-frame stop codon.

homozygosity for the G insertion mutation in the affected individual with both parents shown to be heterozygous carriers (data not shown).

Analysis of ERGIC-53 Expression in Lymphocyte Cell Lines Derived from Patients with Combined Factors V and VIII Deficiency

To determine whether the two mutations identified in the *ERGIC-53* gene resulted in loss of ERGIC-53 expression, immunofluorescence of EBV-immortalized B-lymphocytes derived from patients 2-1 (Middle Eastern Jewish, G insertion mutation), 6-3 (Sephardic Jewish, splice donor mutation), and a control individual was performed. Cells were stained with a monoclonal antibody to ERGIC-53 (Schweizer et al., 1988), a monoclonal antibody to a control ER protein, p63 (Schweizer et al., 1993), and a negative control antibody (Figure 5). All three cell lines showed positive cytoplasmic staining with the p63 antibody. Though a similar cytoplasmic pattern of staining was seen in the normal cells with the ERGIC-53 antibody, no evidence of ERGIC-53 protein expression was detected in either patient cell line. Western analysis of cell extracts prepared from the EBV-immortalized B-lymphocytes using an anti-ERGIC-53 rabbit polyclonal antiserum also confirmed complete absence of ERGIC-53 immunoreactive protein (Figure 6). These results indicate that both the G insertion mutation and the splice donor mutation result in complete loss of immunologically recognizable ERGIC-53 expression. SDS polyacrylamide gel electrophoresis and Coomassie staining detected no obvious differences in protein content between conditioned media of ERGIC-53 deficient and control EBV-immortalized cell lines (data not shown).

Discussion

The demonstration of homozygosity for an inactivating mutation in all of the affected patients from nine families

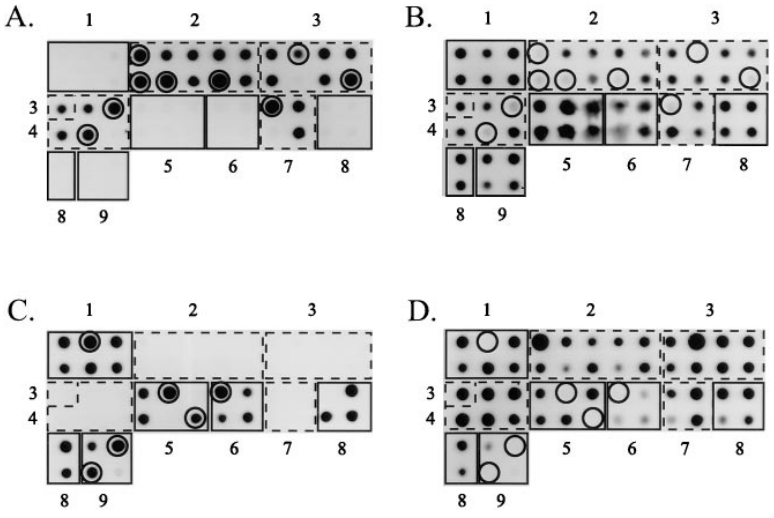

Figure 4. Allele-Specific Oligonucleotide Hybridization Analysis of Combined Factors V and VIII Deficiency Families

ERGIC-53 PCR products derived from members of all nine Sephardic and Middle Eastern Jewish families originally reported in Nichols et al. (1997) were spotted onto nylon membranes and hybridized with oligonucleotides specific for either the G insertion mutation (A), the splice donor mutation (C), or the corresponding normal alleles (B and D, respectively). Grid lines on the filters demark the families, labeled 1–9 (dashed lines, Middle Eastern Jewish; solid lines, Sephardic Jewish). Affected patients are indicated by circled dots with other dots corresponding to unaffected siblings or obligate carrier parents. All affected individuals from the Middle Eastern Jewish families (2, 3, 4, and 7) are homozygous for the G insertion mutation, with all unaffected family members either heterozygous or homozygous for the normal allele. Though negative for the G insertion, all the affected individuals from the Sephardic Jewish families (1, 5, 6, 8, and 9) are homozygous for the splice donor mutation.

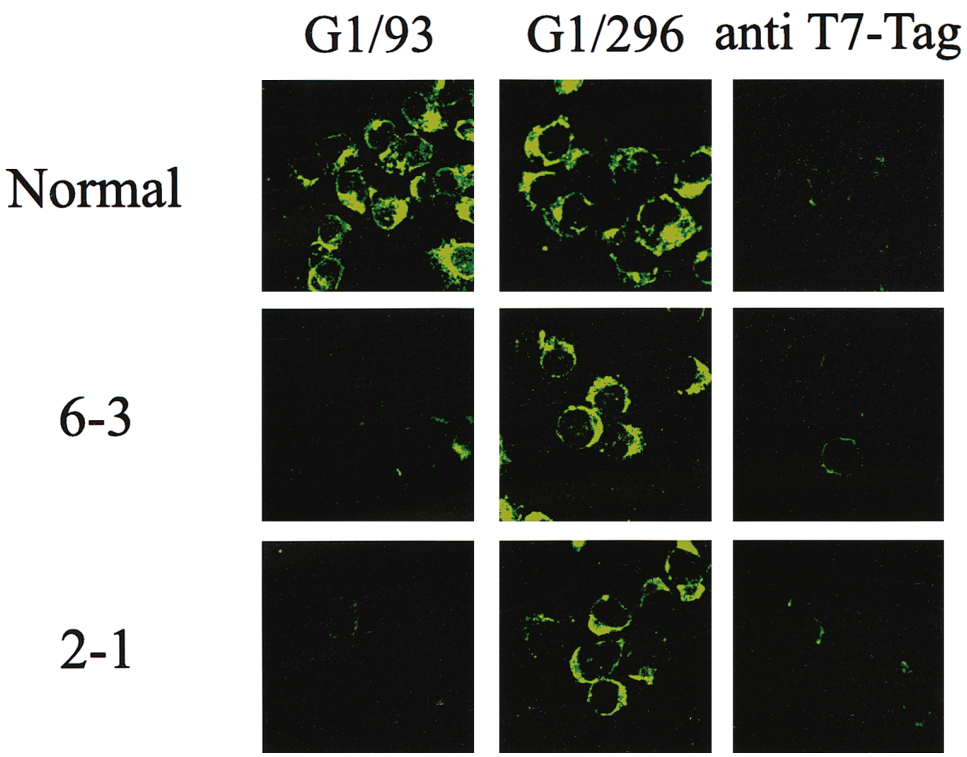

Figure 5. Immunofluorescence Analysis for ERGIC-53 in EBV-Immortalized Lymphocytes Established from Patients with Combined Factors V and VIII Deficiency

EBV-immortalized lymphocytes established from individuals 6-3, 2-1 (See Figure 2), and a control individual were stained with a monoclonal antibody to ERGIC-53 (G1/93), a monoclonal antibody to p63 (G1/296), or a negative control antibody (monoclonal anti T7-Tag). All three cell lines demonstrate a similar pattern of positive staining with G1/296. In contrast, no G1/93 staining is seen in cells derived from the two combined factors V and VIII deficiency patients.

clearly establishes *ERGIC-53* as the gene responsible for combined factors V and VIII deficiency. This surprising finding identifies the molecular mechanism for this disorder as a defect in a common pathway for the intracellular trafficking of these two blood coagulation factors. In addition, these data demonstrate a role for a protein within the ER–Golgi intermediate compartment in the transport of a specific subset of secreted glycoproteins.

The Function of ERGIC-53 and the ER–Golgi Intermediate Compartment

ERGIC-53 was first identified as a 53 kDa protein recognized by the monoclonal antibody, G1/93 (Schweizer et al., 1988). This antibody specifically labels pleomorphic membrane structures between the ER and Golgi, which are collectively referred to as the intermediate compartment or vesicular-tubular cluster (VTC) (Schweizer et al., 1990; Bannykh and Balch, 1997). A number of studies suggest that these structures mark a distinct compartment in the transport between ER and Golgi and that this is the site of a sequential transition from COPII to COPI coated vesicles (Scales et al., 1997).

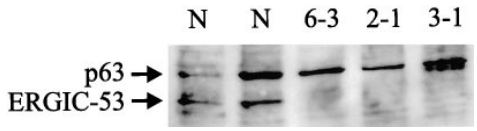

Figure 6. Western Analysis of EBV-Immortalized Lymphocytes

Cell extracts of EBV-immortalized lymphocytes from two control individuals (N), and three combined factors V and VIII deficiency patients (6-3, 2-1, and 3-1; see legend to Figure 2) were fractionated on a reducing SDS-polyacrylamide gel, tranferred to nitrocellulose, and probed sequentially for ERGIC-53 followed by p63. All five cell lines demonstrate a similar pattern of positive staining for p63. In contrast, no ERGIC-53 staining is seen in cells derived from the three combined factors V and VIII deficiency patients.

ERGIC-53 is a type 1 transmembrane protein that exists as homodimers and homohexamers (Schweizer et al., 1988), has homology to leguminous lectins (Fiedler and Simons, 1994), and exhibits mannose-selective and calcium-dependent binding (Arar et al., 1995; Itin et al., 1996). Its amino acid sequence also contains determinants for anterograde transport and a dilysine ER retrieval signal, leading to constitutive recycling between the ER, intermediate compartment, and Golgi (Lippincott-Schwartz et al., 1990; Schindler et al., 1993; Itin et al., 1995). ERGIC-53 is identical to a mannose-specific membrane lectin isolated from human monocytes, MR60 (Arar et al., 1995); and close homologs have been identified in rat (p58) (Saraste et al., 1987; Lahtinen et al., 1996), *Xenopus laevis* (Lahtinen et al., 1996), and *Caenorhabditis elegans* (GenBank accession number Z81097). Recent mutagenesis studies suggest that ERGIC-53 interacts directly with the COPII coat component Sec23p, which may direct it to vesicles budding from the ER (Kappeler et al., 1997). An antibody to the ERGIC-53

cytoplasmic tail was recently shown to block the transport of vesicular stomatitis virus glycoprotein from the ER to Golgi and to block recruitment of COPI to the intermediate compartment in permeabilized cells (Tisdale et al., 1997). These studies suggested that ERGIC-53 may be required for the coupled exchange of COPII for COPI coats during segregation of anterograde and retrograde transported proteins and thus may play an essential role in the general transport of glycoproteins through the secretory pathway. In contrast, our data indicate that ERGIC-53 is only required for the efficient secretion of a specific subset of glycoproteins, rather than a more general role in ER-to-Golgi transport. The normal levels of other plasma proteins in combined factors V and VIII–deficient patients demonstrate that ERGIC-53 is not essential for the integrity of the ER or for the intracellular transport of most secreted proteins.

Anterograde transport from the ER has been assumed to be the default pathway for the bulk flow of secreted proteins, with sorting occurring primarily in the Golgi via specific signals that direct retrieval to earlier compartments or forward transport to the lysosome or plasma membrane (Pfeffer and Rothman, 1987; Schekman and Mellman, 1997). However, studies of albumin (Mizuno and Singer, 1993) and vesicular stomatitis virus glycoprotein (Balch et al., 1994) secretion and, more recently, in *S. cerevisiae* (Kuehn and Schekman, 1997) suggest selective packaging of cargo upon export from the ER. Yeast mutants lacking Emp24p, a component of COP II–coated vesicles, are viable but exhibit reduced delivery of several specific proteins to the Golgi, with the transport of other proteins apparently unaffected. These results suggest the presence of a cellular machinery for the specific sorting of protein cargo into ER-derived vesicles (Schimmoller et al., 1995). Mutations in the V-snare Sec22p or alterations in the level of either the GTPase Sar1p or its guanine nucleotide exchange factor Sec12p that mediate vesicle budding affect the packaging of selective cargo to varying degrees (Campbell and Schekman, 1997). In another recent study, analysis of a panel of mutant alleles in *SEC21*, a component of *S. cerevisiae* COPI, identified several variants that exhibit striking, cargo-selective ER-to-Golgi transport defects. The authors suggest that COPI may play an indirect role in anterograde transport, perhaps by mediating retrieval of other factors that are required for the packaging of selected proteins into COPII vesicles leaving the ER (Gaynor and Emr, 1997).

The results of our study suggest that ERGIC-53 may be serving a similar function for a specific subset of proteins in mammalian cells, including coagulation factors V and VIII. ERGIC-53 could interact directly with these target proteins, directing them to COPII vesicles budding from the ER. Alternatively, ERGIC-53 could facilitate retrieval of other factors from post-ER compartments, which in turn direct FV and FVIII for selective packaging in the ER.

Potential Interactions of ERGIC-53 with FV and FVIII

Are factors V and VIII the only proteins that require ERGIC-53 for efficient transport through the secretory pathway? In addition to the abnormalities identified in patients with combined factors V and VIII deficiency, it seems likely that a defect in at least a limited subset of other proteins may also be present. Perhaps the corresponding reductions in level for these latter proteins are more subtle. Alternatively, levels in the range of 5%–30%, which in the case of FV and FVIII result in significant bleeding, may fail to produce a recognizable phenotype for other proteins regulated through this pathway.

It is important to note that the two mutations identified in this report both appear to result in complete loss of ERGIC-53. Taken together with the observation of residual FV and FVIII activity in the corresponding patients (Seligsohn et al., 1982), these results suggest that ERGIC-53 enhances the efficiency of a sorting event during the transport of these proteins but is not absolutely required for secretion of FV and FVIII.

Is there a unique shared property between coagulation factors V and VIII that target them both to this common secretory pathway? These proteins share up to 40% overall sequence identity across the A and C domains. The plasma protein ceruloplasmin, which also contains similar A domains, exhibits normal levels in these patients (Seligsohn, 1989). As noted above, the tissue-specific patterns and levels of expression of these two proteins differ significantly. In addition, major differences in the intracellular processing and biosynthesis of these two proteins and their interactions with resident ER proteins have been documented (for review, see Kaufman, 1998).

Of note, factors V and VIII both contain a central B domain encoded by a single $\sim$ 3 kb exon. Though only weakly homologous at the amino acid level, the length of these segments is conserved. In addition, both B domains are extensively glycosylated with 18 and 25 potential N-linked sites in FVIII and FV, respectively, and multiple O-linked structures. Recombinant B domain–deleted FV and FVIII retain coagulation activity, and the function of the B domain remains unknown (Toole et al., 1986; Pittman et al., 1994a). Perhaps this shared segment is responsible for the interaction of these two proteins with ERGIC-53, possibly through a lectin-like function, consistent with the apparent affinity of ERGIC-53 for mannose residues (Itin et al., 1996), though inhibition of mannose trimming by inhibitors does not affect the secretion of FV or FVIII (Pittman et al., 1994b).

Human Genetics of Combined Factors V and FVIII Deficiency

Our previous genetic analysis mapped the combined factors V and VIII deficiency gene to the long arm of chromosome 18 and identified two distinct haplotypes, suggesting either two distinct founder chromosomes, or a single ancient founder with a recent split to form the two populations analyzed in our study (Nichols et al., 1997). The identification of distinct mutations corresponding to each haplotype confirm the former hypothesis, documenting a common founder for the combined factors V and VIII deficiency mutation among Sephardic Jews and a different founder for the disease among non-Sephardic Middle Eastern Jews. Future studies of additional combined factors V and VIII deficiency patients in the Mediterranean basin (Seligsohn, 1989; Neerman-Arbez et al., 1997) may provide interesting insights

into the evolutionary history of this disease. Perhaps the apparent increased prevalence of this disorder in this region will be explained by common founder effects, or a selective advantage for heterozygotes.

The unexpected identification of *ERGIC-53* as the gene responsible for combined factors V and VIII deficiency provides evidence for the selective packaging of proteins for transport through the secretory pathway and suggests a central role for the intermediate compartment in this process. Future advances stemming from these findings should enhance the understanding of factors V and VIII biosynthesis, which may lead to improved strategies for the production of recombinant factor VIII as well as gene therapy approaches for the treatment of hemophilia and related bleeding disorders.

Experimental Procedures

Physical Mapping

Yeast Artificial Chromosomes (YACs)

A complete YAC contig spanning the genetic interval D18S849–D18S64 was constructed by PCR and pulsed-field gel electrophoresis (PFGE) analysis. In brief, six YACs clones known to span the interval (Whitehead Institute for Biomedical Research/MIT Center for Genome Research) were obtained from Research Genetics (Huntsville, AL), streaked to single colonies, and cultured as previously described (Chandrasekharappa et al., 1992). Seven of the short tandem repeat markers (STRs) used in the previous genetic linkage analysis (Nichols et al., 1997), as well as four sequence-tagged sites (STSs) and 11 expressed sequence tags (ESTs), were placed on the YAC contig by PCR of total genomic yeast DNA, prepared as previously described (Chandrasekharappa et al., 1992), containing the YAC clone of interest. After agarose plug preparation, individual YAC clones were sized by PFGE and Southern analysis using as probe a 2.7 kb PvuII–Bam HI fragment of pBR322, which also corresponds to the left arm of the pYAC4 vector.

Bacterial Artificial Chromosomes (BACs)

A human BAC library (Research Genetics, Huntsville, AL) was screened by PCR to construct a partial BAC contig across the candidate interval. Thirty-two BAC clones were identified by screening with the STRs, STSs, and ESTs used to construct the YAC contig. Clones of interest were obtained from Research Genetics and cultured as previously described (Kim et al., 1992, 1996; Shizuya et al., 1992). Sizes of BAC clones were determined by PFGE of NotI digested BAC DNA, which separates the 7.3 kb pBeloBAC11 vector from the insert fragment(s). Sequence of BAC clone ends was determined using automated sequence analysis of whole BAC clones by the University of Michigan DNA Sequencing Core after purification of BAC DNA using the Plasmid Maxi Kit (QIAGEN, Santa Clarita, CA) with subsequent phenol/chloroform extractions and ammonium acetate/ethanol precipitation. STSs developed from the BAC end clone sequences were used to assist in contig construction. While a complete, overlapping set of clones was established for the interval D18S1103–D18S64, only a partial contig was completed for the interval D18S849–D18S1103.

Expressed Sequence Tags (ESTs)

Nineteen ESTs cloned from liver libraries were identified from the human transcript map (accessed at The National Center for Biotechnology Information Website) (Schuler et al., 1996) as potentially mapping to the candidate interval. PCR primers were synthesized by the University of Michigan Oligonucleotide Synthesis Core. PCR analysis using EST-specific primer pairs of the clones comprising the YAC and BAC contigs was used to determine those ESTs contained within the candidate interval.

PCR of Reverse Transcribed Lymphocyte RNA

Total RNA was prepared from patient and normal EBV-immortalized B lymphocytes using TRIZOL Reagent (GIBCO BRL). In brief, $\sim 40 \times 10^6$ cells were pelleted and resuspended in 4 ml TRIZOL Reagent. After incubating at room temperature for $\geq$5 min, 1/5 vol of chloroform was added followed by vigorous shaking and incubation for 2–3 min. The aqueous phase was removed after centrifugation and precipitated with volume 100% isopropanol. The RNA was pelleted by centrifugation and washed once with 75% isopropanol. After air-drying, the RNA was dissolved in DEPC-treated deionized water. Concentration was determined by spectrophotometry. Reverse transcriptase (RT) reaction was performed using 1 μg total RNA as template in a 40 μl reaction containing 2 μg oligo dT, 625 μM each dNTP, 1$\times$ RT buffer (50 mM Tris-Cl [pH 8.5], 8 mM $MgCl_2$, 30 mM KCl, 1 mM DTT), and 25 U AMV reverse transcriptase (Boehringer Mannheim). After incubation at 41°C for 1 hr, PCR was performed using 10 μl of the RT reaction in a 50 μl reaction containing 100 ng of the appropriate forward and reverse primer (See Table 1), 1$\times$ adjustment buffer (44 mM KCl, 0.01% gelatin, 0.1% Triton X-100), 100 μM each dNTP, and Taq polymerase. Cycling was performed using an MJ Research PTC-100 96V thermocycler (Watertown, MA) with 1 min each of denaturation, annealing, and elongation for 35 cycles. PCR products were electrophoresed through 2% agarose and visualized by staining with ethidium bromide. Sequence of PCR products was determined either manually using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science, Arlington Heights, IL) or using automated sequence analysis by the University of Michigan DNA Sequencing Core after purification using the QIAquick PCR Purification Kit (QIAGEN, Santa Clarita, CA)

DNA PCR and Allele-Specific Oligonucleotide (ASO) Hybridization

Twenty nanograms of total genomic DNA, prepared as previously described (Nichols et al., 1997), was amplified by PCR in a 20 μl reaction as previously described (Nichols et al., 1996) using the appropriate forward and reverse primers (See Table 1). Cycling conditions were 1 min each of denaturation, annealing, and elongation for 35 cycles with a final extension of 5 min. After PCR, ASO hybridization analysis was performed essentially as described (Cooney et al., 1991) using oligonucleotides corresponding to both normal and mutant sequences (Table 1).

Immunofluorescence

Immunofluorescence of EBV-immortalized B lymphocyte cultures established using standard methods (Sugden and Mark, 1977) from patients 2-1 and 6-3 (Nichols et al., 1997) and two control individuals was performed as follows. Immortalized cells were resuspended in 5% BSA in phosphate-buffered saline (PBS), and 1×10^5 were applied to slides using a Cytospin3 (Shandon, Pittsburgh, PA). After air drying for 30 min on ice, the cells were fixed in 4% paraformaldehyde in PBS for 20 min on ice. The slides were rinsed once in PBS for 5 min, once in 50 mM NH_4Cl in PBS for 20 min, and once in double distilled deionized water, all at room temperature. The cells were permeabilized with absolute methanol on ice for 20 min, rehydrated with three 5 min rinses in PBS at room temperature, and blocked for 20 min at room temperature in 2% normal goat serum, 0.05% Triton X-100, and 0.05% Tween 20 in PBS. After blocking, the cells were incubated with a 1/50 dilution of monoclonal anti-ERGIC-53 antibody G1/93 (Schweizer et al., 1988), a 1/50 dilution of monoclonal anti-p63 antibody G1/296 (Schweizer et al., 1993), or a 1/200 dilution of a negative control antibody (monoclonal anti-T7 tag, Novagen, Madison, WI) in blocking solution overnight at 4°C. After overnight incubation, the slides were washed three times at room temperature in PBS. The cells were incubated in a 1/200 dilution in blocking solution of fluorescein-conjugated goat IgG fraction to mouse IgG (ICN, Aurora, OH) for 1 hr at room temperature. After three 5 min washes in PBS, the slides were air dried and mounted with Immunofluore (ICN, Aurora, OH). The cells were visualized using a Meridian Instruments Ultima confocal microscope (Meridian Instruments, Okemos, MI).

Western Blotting

Cell extracts were fractionated on 8% reducing SDS-polyacrylamide gel as previously described (Dorner and Kaufman, 1990). Western blot analysis was performed with a polyclonal anti-ERGIC-53 rabbit antiserum/HRP-conjugated goat anti-rabbit antiserum (Boehringer

Mannheim Biochemicals, Indianapolis, IN) followed by a monoclonal anti-p63/HRP-conjugated goat anti-mouse antiserum (Boehringer Mannheim Biochemicals, Indianapolis, IN). Visualization was performed using the ECL Western blotting detection reagents (Amersham Life Science, Arlington Heights, IL).

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