The Carboxyl-Terminal Region of Factor IX Is Essential for Its Secretion†

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ABSTRACT: The carboxyl-terminal region of factor IX (residues 403−415) contains several natural mutations which result in mild to severe forms of hemophilia B. A battery of factor IX minigene expression vectors carrying various mutations in the C-terminal region were constructed and studied by transient expression assay using HepG2 cells. Mutations included in this study are Y404P, I408N, T412N, T412S, T415G, T415S, T415L, and T415R as well as five selected naturally occurring mutations in the region, R403Q, R403W, Y404H, W407R, and T412K. In comparison to the normal factor IX, these mutations neither significantly affected the factor IX mRNA level nor affected the stability of the secreted factor IX in the culture medium but did decrease to various extents the intracellular and secreted factor IX protein levels as quantified by enzyme-linked immunosorbent assay. T415L, T415S, and T415R showed only minor reductions in the intracellular and minor to moderate reductions in the secreted factor IX levels. T415G showed only minor reduction in the intracellular factor IX level but substantial reduction in the secreted levels. R403Q, R403W, and T412S showed moderate reductions in both intracellular and secreted factor IX levels. Y404H, Y404P, W407R, I408N, T412K, and T412N also showed minor to moderate reductions in the intracellular factor IX levels but very severe reductions in the secreted factor IX level. In one stage clotting assays, secreted factor IX mutants showed specific activities very similar to that of the normal factor IX, suggesting that the carboxyl-terminal region is neither directly involved in the tenase complex formation required for factor X activation nor involved in the activation of factor IX itself. In comparison to the normal factor IX, secreted levels of factor IX mutants with mutations R403Q, Y404H, W407R, and T412K were also very similar to the plasma levels reported for the hemophilia B patients carrying the same mutations. Treatment of cells with proteasome inhibitors (ALLM and ALLN) added to the culture medium at 50 μM resulted in drastic increases of the intracellular mutant factor IX to the levels equivalent to that of the normal factor IX, while the secreted factor IX levels were little or only marginally affected. With a higher concentration of the inhibitors (100 μM), however, both the intracellular and secreted mutant factor IX were fully elevated to the normal factor IX levels. Intracellular and secreted levels of the normal factor IX were little affected by the low inhibitor concentration and only marginally, if at all, at the higher concentration ($\leq$10%). Serine protease inhibitors did not significantly affect intracellular and secreted factor IX levels. These results indicate that the carboxyl-terminal region plays a critical role in the cellular secretion of factor IX and that the mutant factor IX proteins carrying specific mutations in this region are subjected to efficient elimination by the proteasome protein degradation mechanism. Furthermore, these results strongly support that HepG2 cells can be utilized as a robust *in vitro* assay system for studying factor IX biosynthesis, well mimicking the *in vivo* phenomenon.

Factor IX is a plasma glycoprotein which plays an essential role in the middle phase of the blood coagulation cascade (Kurachi et al., 1993; Saito, 1991). Its deficiency or reduced functional level results in an abnormal bleeding disorder, hemophilia B, with various levels of severity (Kurachi et al., 1993; Giannelli et al., 1994). Since its normal gene was isolated and completely sequenced a decade ago (Yoshitake et al., 1985), almost 1400 abnormal human factor IX genes have been studied at the molecular level, producing a wealth of knowledge on the structure−function relationship of the factor IX gene and protein (Giannelli et al., 1994, 1996). This makes factor IX one of the most thoroughly studied mammalian proteins.

The factor IX database (Giannelli et al., 1994, 1996) shows that mutations in two regions, one spanning aa[1] sequence 191−198 and the other spanning the carboxyl-terminal (C-terminal) region aa 403−415 and a significant number of randomly scattered mutations present a unique class of hemophilia B. Most of these mutations display severely reduced factor IX antigen and activity levels in the circulation. The sequence region aa 191−198 is a part of the hydrophobic core of the catalytic subunit. Understandably, most, but not all of the randomly distributed mutations are nonsense mutations or mutations at splicing sites (Giannelli et al., 1994). The resulting phenotype is analogous to that

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[1] Abbreviations: aa, amino acid; C-terminal, carboxy terminal; PCR, polymerase chain reaction; DMEM, Dulbecco's modified eagle medium; FCS, fetal calf serum; ALLM, *N*-acetyl-L-leucinyl-L-leucinyl-L-methioninal; ALLN, *N*-acetyl-L-leucinyl-L-lencinyl-L-norleucinal; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N-tosyl-L-lysyl chloromethyl ketone; CNBr, cyanogen bromide; ELISA, enzyme-linked immunosorbent assay; ds, double stranded; ss, single stranded; nt, nucleotide; bp, base pairs; PBS, phosphate-buffered saline; ER, endoplastic reticulum.

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observed for hemophilia B patients with mutations in the promoter region of the factor IX gene (Kurachi et al., 1993, 1994; Hirosawa et al., 1990), though the mutations in the coding regions of factor IX may not be directly involved in the transcriptional regulation of the gene. Mutations, particularly nonconservative mutations, in the region spanning aa 191−198 were conceived to disrupt the intact conformation of factor IX. It has been more difficult to speculate about the role of the C-terminal region because of its unique location in the factor IX molecule and absence of any known target sites for co- and post-translational processing or modifications. Recently, the X-ray structure of human factor IX was reported (Brandstetter et al., 1995), showing the C-terminal region in an amphipathic α-helical structure. Little is known about the molecular mechanisms, of how those mutations confer a unique phenotype displaying greatly reduced plasma factor IX levels.

Abnormal structures and post-translational modifications of various other proteins, such as protein C and prothrombin, are known to affect their normal secretory process, resulting in reduced levels of the protein in the circulation (Halban & Irminger, 1994; Grinnell et al., 1991; Sugahara et al., 1994; Watzke et al., 1991; Yamamoto et al., 1992; Pittman et al., 1994). Recently, rapid intracellular degradation of protein C, which was produced by warfarin-treated cells, was reported to be due to the "quality control" mechanism of the protein trafficking pathway in the cells (Tokunaga et al., 1995).

In this paper, we describe mutagenesis studies on the carboxyl-terminal region of factor IX, demonstrating that this region plays an important role in the efficient secretion of normal factor IX and that mutations in this region lead to rapid elimination of the mutant proteins primarily by the proteasome mechanism.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes and other DNA modification enzymes were purchased from Life Technologies, Inc. or New England Biolabs. [^{35}S]dATP, [^{32}P]dCTP, Megaprime labeling kit, and the Sculptor *in vitro* mutagenesis kit were purchased from Amersham Inc. DNA sequencing kit (Sequenase version 2.0) was purchased from United States Biochemical. PCR supplies including Vent$_R$ DNA polymerase and M13mp18 phage vector were obtained from New England Biolabs. Qiagen Plasmid kit was obtained from Qiagen Inc. Fast Track poly(A)$^+$RNA isolation kit and Top10 F′ cells were purchased from Invitrogen Corp. DMEM, FCS for tissue culture medium, and sheared herring sperm DNA were obtained from GIBCO Life Technologies. Vitamin K (AquaMephyton) was obtained from Merck Sharp & Dohme. Maximum strength Nytran and PVDF filters were obtained from Schleicher and Schuell Co. and BioRad, respectively. Monoclonal antibody against human factor IX (AHIX 5041) was purchased from Hematologic Diagnosis Inc., and polyclonal anti-human factor FIX was a gift of Dr. Kenneth Smith (University of New Mexico). Human factor IX-deficient plasma and a pooled normal plasma were obtained from George King Bio-Medical, Inc. Chamber slides and Immuno-histological staining kit were purchased from Nunc and Zymed Laboratories Inc, respectively. Chemiluminescence Western blotting kit and Complete protease inhibitor cocktail tablets were obtained from Boehringer Mannheim, Inc. ALLM, ALLN, TPCK, and TLCK were obtained from Sigma Chemical. RNR18 probe was obtained from ATCC.

FIGURE 1: Schematic structure of factor IX minigene expression vector, p-416hFIXm1. Open boxes, hatched boxes, and solid boxes indicate 5′ or 3′ flanking sequences, 5′ or 3′ UTRs, and coding regions of factor IX cDNA, respectively. Solid and broken lines indicate the retained and deleted regions of the intron I sequence, respectively. Numbers indicate the relevant nucleotide positions cited in the text. Arrows and asterisks indicate the 5′ upstream transcriptional start site and the polyadenylation site, respectively.

Cells. HepG2 cells, a human male origin hepatoma cell line (Knowles et al., 1980; Fair & Hahnak, 1984; Darlington et al., 1987), were used for transient expression assays as previously described (Kurachi et al., 1995). Cells were grown and maintained in DMEM supplemented with 10% FCS and antibiotics (penicillin and streptomycin) at 37 °C under 5% CO_2 in a humidified incubator. FCS used in culturing cells for factor IX assays was pretreated with barium sulfate to eliminate factor IX (Yao & Kurachi, 1992).

Oligonucleotide Synthesis. Mutagenic oligonucleotides (summarized in Table 1) were chemically synthesized using an automated DNA synthesizer (Applied Biosystems, model 394) at the University of Michigan Biomedical Research Core Facility. Each mutagenic oligonucleotide was designed to generate multiple single as well as compounded mutations. Nucleotides in parentheses indicate intended mutations.

Template Construction and Mutagenesis. The factor IX genomic nt sequence numbering system was used as previously published (Yoshitake et al., 1985). A 762 bp fragment, corresponding to the region sandwiched by an internal *Ava*I site in exon 6 at nt +20 517 and a *Bam*HI site derived from a linker attached at nt +32 691 of the factor IX gene (Figure 1), was amplified by PCR from the 3′ half of a factor IX minigene expression vector p-416FIX m1. The 5′ forward PCR primer used was 5′-GACCGAATTCTCGGGTTGT-TGGTGGAG-3′, which contained an *Eco*RI linker sequence 5′ to an *Ava*I sequence corresponding to the above mentioned internal site. The 3′ reverse PCR primer used was 5′-CTCTGGATCCTGTTAATTTTCAATTCC-3′, which contained a *Bam*HI site sequence. The amplified *Eco*RI-*Ava*I/*Bam*HI fragment was digested with *Eco*RI and *Bam*HI and inserted into a ds form of M13 phage vector (M13mp18) at the *Eco*RI/*Bam*HI sites, thus producing the mutagenesis template vector M13FIX (*Ava*I/*Bam*HI). The coding strand of the FIX template was the single (+) strand of the resultant phage DNA.

Mutagenesis was carried out using the Sculptor *in vitro* Mutagenesis System according to the manufacturer's instructions, with the ss phage DNA of M13FIX(*Ava*I/*Bam*HI) as the template and the mutagenic oligonucleotides containing various degenerated nucleotide sequences (Table 1). These oligonucleotides were designed to generate multiple mutant factor IX sequences in each mutagenesis reaction. Mutant phage clones obtained were then sequenced through the mutagenized sites of the C-terminal coding region using the Sequenase Version 2.0 Sequencing Kit and the 3′ reverse PCR primer (above mentioned) as the sequencing primer. Double stranded M13FIX(*Ava*I/*Bam*HI) DNAs from these clones were prepared using the alkaline lysis method

Table 1: Mutagenic Oligonucleotides[a]

oligonucleotide	sequence
FIX403/404/405	5′-CCAGTTGA(C/A)AT(A/G)C(C/T)(G/A)GGATAC-3′
FIX407/408	5′-TTTTTCCTTA(T/A)TCC(A/C/G)GTTGAC-3′
FIX412/414/415	5′-TTAAG(T/C)G(A/G)GCTTT(G/T/A)TTTTTTC-3′
FIX412	5′-AGTGAGCTT(C/A)(C/A/T)TTTTTTCCTT-3′
FIX415	5′-TTTCATTAA(C/T)(T/G/C)GAGCTTTGTT-3′

[a] Oligonuceotides were named with the corresponding residue number(s) intended for mutagenesis. Nucleotides in parentheses indicate possible mutations.

(Sambrook et al., 1989). *Ava*I/*Bam*HI fragments were isolated from clones with mutations and inserted into a previously constructed p-416FIXm1 vector (Kurachi et al., 1995), replacing its *Ava*I/*Bam*HI sequence (Figure 1). The ligated plasmid DNAs were transfected into competent Top10 F′ cells, and ampicillin resistant colonies were obtained. The *Ava*I/*Bam*HI insert fragments of p-416FIXm1 vector DNAs prepared from these cells were then completely sequenced, including ligation sites to confirm the presence of intended mutations and no other unwanted mutations. Large-scale preparations (300−500 μg) of the mutated expression vectors were carried out using the Qiagen-tip 500 plasmid kit. These DNA preparations could be used adequately without further purification for transfection of HepG2 cells in transient expression assays (Briggs et al., 1993).

CNBr Cleavage, Western Blot Analysis, and High-Performance Liquid Chromatography. CNBr cleavage was carried out according to the standard method (Gross, 1967). Human factor IX (100 μg) was incubated with CNBr (10 mg) in 0.1 mL of 10% trifluoroacetic acid at room temperature for 24 h. A portion of the CNBr-cleaved factor IX sample (150 ng) was then subjected to polyacrylamide gel electrophoresis (7.5% gel) under the denaturing condition (0.1% sodium dodecyl sulfate) with and without mercaptoethanol, followed by blotting with PVDF filters. The filters were then incubated with monoclonal antibody (AHIX5041) or polyclonal anti-human factor IX antibody, and protein bands transferred to the filters were visualized by chemiluminescence immunostaining (Thorpe et al., 1989). Polypeptide fragments generated by CNBr-cleavage were also separated with a high-performance liquid chromatography (HPLC) equipped with C-4 column (2.1 × 250 mm) (Applied Biosystems) at the Biomedical Research Protein Core facility of this campus. Isolated fragments were analyzed for their amino-terminal sequences with an automated sequencer (Applied Biosystems, model 473A) at the Protein Core.

ELISA and Factor IX Clotting Activity Assay. Quantitation of produced factor IX proteins and polypeptide fragments generated by CNBr cleavage of factor IX was carried out by ELISA using AHIX5041 as the catching antibody and alkaline phosphatase-linked polyclonal anti-mouse IgG as the detecting antibody as previously described (Kurachi et al., 1995). The ELISA method was highly refined and was able to reproducibly quantify factor IX at about 0.5 ng/mL with an extreme specificity for human factor IX, permitting us to reproducibly quantify secreted and intracellular human factor IX.

Clotting activity (activated partial thomboplastin time, aPTT) of human factor IX was assayed by one-stage clotting assay using human factor IX deficient plasma as previously described (Kurachi et al., 1995). Serially diluted pooled normal human plasma samples were used for generating both ELISA and clotting activity standard curves. All samples were routinely assayed in duplicates at two different dilutions.

Transient Factor IX Expression Assay. Transient expression assays of the factor IX minigene expression vectors with and without mutations were carried out with HepG2 cells using the high-efficiency calcium phosphate−DNA precipitation method with β-galactosidase as internal standard (Kurachi et al., 1995). This method gives approximately 5% transfection efficiency for HepG2 cells under the conditions used and was sufficient to reproducibly quantify factor IX produced as previously described.

Intracellular factor IX levels were determined as follows. After 60 h of post transfection, cells were washed twice with excess volume of PBS and quickly collected by scraping into a total of 10 mL of PBS added with 200 μL of Complete protease inhibitor cocktail (1 tablet/mL). Cell pellets were collected by a brief centrifugation and lysed by repeated freeze and thawing (three times) in 150 μL of cell lysis buffer (250 mM Tris-HCl, pH 7.8) containing proteinase inhibitor cocktail (1 tablet/50 mL) with mechanical pipetting up and down. Cell protein extracts were obtained by centrifugation at 10 000 rpm for 15 min at 4 °C, followed by spectroscopic quantitation of the total amount of proteins. Routinely, serially diluted samples of the extracts (8- and 16-fold dilutions) were assayed by ELISA for intracellular human factor IX. Sheared herring sperm DNA and p-416FIXm1 (the normal factor IX minigene) were used as the mock and the normal factor IX expression level controls, respectively. Values obtained for the herring sperm DNA mock control were subtracted from those of the normal and mutant factor IX expression vectors. Unless otherwise mentioned, all expression vectors were assayed in duplicate in at least five independent experiments, and averages of the results with standard deviations are presented.

Stability Assay of the Secreted Proteins in the Culture Medium. Aliquots (2 mL) of the culture medium of cells transfected with factor IX expression vectors were transferred in duplicates into fresh, sterile tubes and briefly centrifuged to remove cell debris. One set of tubes (0 h samples) was immediately frozen at −80 °C until further use in assaying clotting activities and antigen levels. The other sets (24 h samples) were incubated for 24 h at 37 °C in a humidized incubator and frozen. All these samples were then thawed under the same conditions and subjected to assaying factor IX clotting activity and antigen levels. Relative levels of activity and antigen of mutant factor IX proteins after 24 h incubation over those at 0 h incubation were obtained and compared with the levels obtained for the normal factor IX protein.

Northern Blot Analysis. Factor IX mRNA produced in the transient assays was analyzed by Northern blot analysis and quantified as previously described (Kurachi et al., 1995). The *Ava*I/*Bam*HI fragment (762 bp) containing the 3′ half

Table 2: Factor IX Protein and Activity Levels Produced by Mutant FIX Constructs in HepG2 Cells

mutation	factor IX protein level (%)[a] intracellular	factor IX protein level (%)[a] secreted	factor IX activity (%)[a] secreted	specific activity (%)[b]	group[c]
wild type	100	100	100	100	
R403Q	61 ± 7.1	59 ± 5.1 (60)[d]	54 ± 8.1 (24)[d]	92 (40)[d]	III
R403W	52 ± 5.7	54 ± 7.8[e]	47 ± 14.2[e]	87	III
Y404H	41 ± 6.4	7 ± 2.6[e]	(4)[d]		IV
Y404P	52 ± 4.8	ND[f]			IV
W407R	58 ± 8.4	ND[f] (2)[d]	(<1)[d]		IV
I408N	41 ± 7.2	ND[f]			IV
T412K	86 ± 2.1	<2 (1)[d]	4.2 ± 2.8 (3)[d]		IV
T412N	66 ± 8.5	ND[f]			IV
T412S	69 ± 9.5	50 ± 6.8	49 ± 12.5	98	III
T415G	88 ± 4.1	27 ± 5.9	21 ± 11.6	78	II
T415L	89 ± 5.7	85 ± 8.9	74 ± 10.2	87	I
T415S	74 ± 5.9	60 ± 4.2	59 ± 9.7	98	I
T415R	108 ± 14.1	63 ± 9.7	51 ± 16.4	81	I

[a] Value represents mean factor IX levels ± SD (five independent assays). Levels of normal factor IX are defined as 100%. [b] Specific activity was calculated by dividing secreted factor IX activity level with secreted factor IX protein levels. Only those secreted at reproducibly measurable levels are shown. [c] Classification of factor IX protein levels: group I, no or small reductions in intracellular and secreted levels; group II, small or reductions in intracellular levels with substantial reductions in secreted levels; group III, moderately reduced intracellular and secreted levels; group IV, small to moderate reductions in intracellular levels with severe reductions in secreted levels. [d] Values in parentheses are those reported for hemophilia B patients (Giannelli et al., 1994, 1996). [e] Values not available for the specified natural mutation (Giannelli et al., 1994, 1996). [f] ND indicates not detectable.

of the factor IX cDNA was radiolabeled with ^{32}P and used as the hybridization probe. After quantifying the mRNA bands with PhosphorImager (Molecular Dynamics, Inc., model 400E), filters were stripped of the factor IX probe, and rehybridized with an internal RNA control probe, ^{32}P-labeled RNR18 (18S ribosomal RNA cDNA). RNR18 RNA bands were quantified in a similar manner as described for factor IX mRNA.

Immunostaining Analysis of Cells. Immunostaining of HepG2 cells were carried out as we previously described (Yao et al., 1991) with minor modifications using 1:200 diluted AHIX 5041 as the primary antibody.

Treatment of Cells with Protease Inhibitors. All the conditions for cell culture and transfection were the same as described above for transient expression assay, except for the following modifications. On day 2 post cell transfection, a mixture of proteasome inhibitors (ALLM and ALLN) or serine protease inhibitors (TPCK and TLCK), which were dissolved in dimethyl sulfoxide, were added to the culture medium at a final concentration of 50 or 100 μM, and incubated for another 48 h. Control experiments, which were run simultaneously with the sample experiments, used the same amount of dimethyl sulfoxide without containing the inhibitors. At the end of incubation, culture medium and cells were collected and used for quantifying factor IX protein by ELISA as mentioned above.

RESULTS

Construction of Mutant Factor IX Expression Vectors. Mutagenesis reactions were carried out by using the oligonucleotides listed in Table 1. Mutations generated are routinely represented by the normal amino acid sequence residue on the left followed by the residue number and the mutant amino acid residue as in R403Q.

As shown in Table 2, 13 mutations were selected out of a total of 28 mutations generated as best encompassing the C-terminal region including as many naturally occurring mutations as possible. These included mutations at amino acid residues R403, Y404, W407, I408, T412, and T415. Among these, five mutations (R403Q, R403W, Y404H, W407R, and T412K) were naturally occurring mutations found in hemophilia B patients. Y404P was a spontaneous mutation found in a mutagenesis experiment. Four mutations at T415 were included in the testing to examine the positional importance at the C-terminus, where no natural single base mutations were reported.

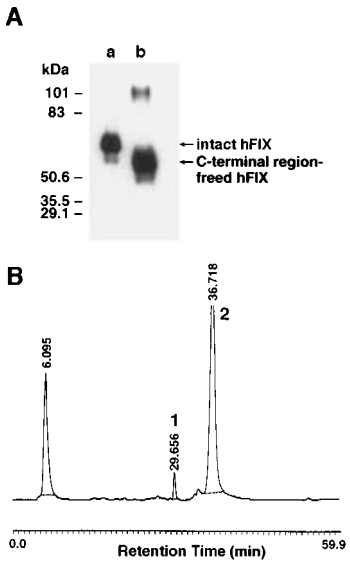

FIGURE 2: Regional localization of the AHIX5041 epitope. Panel A: Western blot analysis of the CNBr-cleaved human factor IX fragments. Polypeptide bands were identified with AHIX5041. Lane a, loaded with the intact factor IX; Lane b, loaded with CNBr-cleaved factor IX fragments.

Determination of the Epitope Site for AHIX5041. The plasma factor IX has three methionine residues (residue 19, 348, and 391), suggesting that upon its CNBr cleavage, the C-terminal region (residue 392−415) should be freed in the nonreducing condition from the rest of the three anticipated fragments held together with disulfide bridges. Western blot analysis of the CNBr cleavage products was consistent with this prediction (Figure 2A). AHIX5041 recognized the intact factor IX (control) as well as the factor IX fragment generated by CNBr-cleavage, which is missing the C-terminal region (24 amino acid residues in length). The

C-terminal region fragment was isolated by HPLC (Figure 2B, peak 1), free from the rest of the molecule (peak 2). Amino-terminal sequence analysis of the peak 1 (100 pmol) showed a clean single sequence: Lys (48.6), Gly (41.2), Lys (41.2), Tyr (37.6), Gly (32.4), Ilu (34.0), Tyr (34.8) (numbers in parenthesis: pmole), confirming its identity with the C-terminal region of factor IX. ELISA with AHIX5041 showed no appreciable recognition of the C-terminal fragment (optical density reading at 405 nm = 0.003), essentially same as the BSA control. The C-terminal regionless factor IX and intact factor IX, however, were strongly recognized at the similar levels (optical density readings at 405 nm after normalization for the required sample dilutions = 2.72 and 2.85, respectively). These observations indicated that the C-terminal region is not involved in the epitope site of AHIX5041.

Intracellular and Secreted Factor IX Levels. Results of transient expression assays of the mutant factor IX minigene constructs are summarized in Table 2. Intracellular or secreted levels of mutant factor IX proteins produced are presented as relative values to those for the normal factor IX. In a typical transient assay of the normal factor IX expression vector, the secreted and intracellular factor IX levels produced were 278 ng/10 mL of culture medium and 52.1 ng/mg of protein extracts, respectively. The ELISA method used in this study could reproducibly quantify the levels of either secreted or intracellular factor IX as low as ~2% of the corresponding normal factor IX levels. No detectable expression of the endogenous factor IX gene in HepG2 cells made this assay system highly reliable and accurate.

T415L, T415S, and T415R, which were arbitrarily categorized as group I, gave no or only small reductions in intracellular factor IX levels maintaining 108−74% of the normal and small to moderate reductions in secreted factor IX levels (85−60% of the normal). T415G (group II) gave small reductions in intracellular factor IX levels, and substantial reductions in secreted levels, maintaining 88% and 27% of the normal, respectively. R403W (natural mutant), R403Q (natural mutant), and T412S in group III showed moderate reductions in both intracellular and secreted factor IX levels down to 69−52% and 59−50% of the normal, respectively. Y404H (natural mutant), Y404P, W407R (natural mutant), I408N, T412K (natural mutant), and T412N in group IV showed small to moderate reductions in the intracellular factor IX levels (86−41% of the normal), but severely reduced secreted factor IX levels down to the 7% of the normal or not detectable levels. Relative secreted factor IX protein levels found for naturally occurring mutants (R403Q, Y404H, W407R, and T412K) in our culture cell assay system agreed remarkably well with the relative factor IX plasma levels reported for in hemophilia B patients with those mutations. No plasma factor IX protein as well as activity levels were reported for the patient with R403W (Giannelli et al., 1994; 1996).

Indirect Immunostaining of HepG2 Cells. Immunostaining results of the transfected cells showed an absence of intracellular factor IX in cells transfected with herring sperm DNA (A) and its presence in cells transfected with p-416FIXm1 containing the normal factor IX sequence (B), Y404H (C), T412K (D), R403Q (E), or T412S (F) (data not shown). The apparent transfection efficiency, shown in this experiment as the factor IX expressing cell fractions was in a range of approximately 5%, agreeing well with the estimation using the β-galactosidase control. The secreted levels of factor IX observed in this set of experiments were also in good agreement with those summarized in Table 2, obtained from a separate set of experiments.

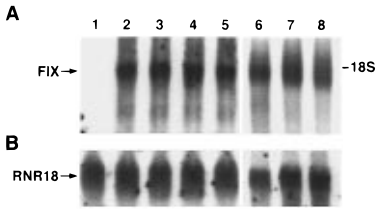

FIGURE 3: Northern blot analysis of HepG2 cells transfected with the normal and mutant factor IX expression vectors. Poly(A)$^+$ RNA samples (2.5 μg per lane) prepared from the cells transfected with various expression vectors were analyzed. Panels A and B show factor IX mRNA bands (FIX) and RNR18 mRNA bands (internal control for the applied RNA amounts), respectively. Lane 1, mock (herring sperm DNA); lanes 2 and 6, p-416hFIXm1 (normal control); lane 3, p-416FIXm1 with Y404H; lane 4, p-416FIXm1 with T412K; lane 5, p-416FIXm1 with T412S; lane 7, p-416FIXm1 with R403Q; Lane 8, p-416FIXm1 with W407R. Lanes 6−8 set is from a separate experiment from the lanes 1−5 set. Mature factor IX mRNAs (1.8 kb in size) are shown by the arrow. Detailed experimental conditions are described in the text.

Clotting Activity of Secreted Factor IX. Clotting activities and specific activities of the secreted mutant factor IX proteins are shown in Table 2, with the reported values of the natural mutations in parenthesis. Clotting activities of all factor IX mutants agreed reasonably well with the secreted level of factor IX proteins within the experimental errors, indicating that the specific activities did not change significantly. The plasma factor IX protein level of 60% was reported for the patient with mutation R403Q in the 1994 mutant factor IX database and was in excellent agreement with the present finding (59%). The value reported in the 1996 database, however, was listed significantly different (126%) without any explanation. The clotting activity for R403Q was 54% in the present study (specific activity = 92%), whereas the reported plasma activity for the same mutant factor IX in a patient was 24% (Giannelli et al., 1994, 1996). The reason for this discrepancy in the clotting activity is not known at the present time.

Stability of Mutant Factor IX Proteins in the Culture Medium. Relative clotting activity levels (levels after 24 h incubation over those at 0 h incubation) of factor IX mutants containing R403Q, R403W, T412S, T415G, or T415S (representatives of groups I, II, and III) were 98, 92, 103, 95, and 91%, respectively (averages of two independent experiments with each sample assayed in duplicate). These were very similar to that found for the normal factor IX (94%). Factor IX antigen levels of these mutants in the medium were in good agreement with the activity levels, ranging 93−102%, which also showed no significant differences from that for the normal factor IX (98%). These results indicated that mutant factor IX proteins were as stable as the normal protein in the culture medium.

Steady-State Factor IX mRNA Levels Produced by the Mutant Factor IX Minigene Constructs. Northern blot analysis of steady-state factor IX mRNA levels for the normal and representative mutant factor IX minigene expression constructs is shown in Figure 3. Intensities of mRNA bands for factor IX with mutations Y404H, T412K, T412S, R403Q, and W407R, which were quantified by PhosphorImager and

Table 3: Effects of Proteasome Inhibitor on Intracellular and Secreted Factor IX Levels[a]

	intracellular FIX protein level (%)			secreted FIX protein level (%)		
mutation/inhibitor	0 μM	50 μM	100 μM	0 μM	50 μM	100 μM
wild type	100	105 ± 5.6	111 ± 8.4	100	103 ± 2.8	109 ± 7.1
R403Q	61 ± 7.1	101 ± 6.4	104 ± 7.8	59 ± 5.1	61 ± 5.7	101 ± 9.9
Y404H	41 ± 6.4	87 ± 11.3	107 ± 12.7	7 ± 2.6	6 ± 2.8	96 ± 8.5
W407R	58 ± 8.4	123 ± 7.8	112 ± 9.2	ND[c]	5 ± 1.4	94 ± 11.3
T412K	86 ± 2.1	104 ± 7.1	92 ± 7.1	ND[c]	6 ± 4.2	116 ± 8.5
T412S	69 ± 9.5	119 ± 12.4	102 ± 11.3	50 ± 6.8	73 ± 7.0	101 ± 9.8
T415S	74 ± 5.9	105 ± 9.9	NA[b]	60 ± 3.8	77 ± 4.2	NA[b]
T415R	108 ± 14.1	118 ± 7.1	NA[b]	63 ± 9.7	54 ± 7.8	NA[b]

[a] Values represent mean factor IX levels ± SD (three to five independent assays). Levels of normal factor IX are defined as 100%. [b] NA indicates not assayed. [c] ND indicates not detectable.

normalized to the internal RNR18 ribosomal RNA controls, were in the range 95−112% of that of the normal factor IX (defined as 100%). These results indicated no significant negative effects of the mutations in the C-terminal region on the transcriptional step as well as the mRNA stability.

Effects of Protease Inhibitors on Factor IX Secretion. Effects of proteasome inhibitors (ALLM and ALLN) (Rock et al., 1994) on the factor IX secretion from cells are summarized in Table 3. With a low concentration of inhibitors (50 μM), intracellular antigen levels of mutant factor IX were dramatically elevated to the normal levels, while that of the normal factor IX remained unchanged. Despite the increased intracellular levels, secreted levels of the mutants R403Q, Y404H, and T415R, as well as the normal, did not change significantly, while marginal increases were observed for mutants W407R, T412K, T412S, and T415S. With a higher inhibitor concentration (100 μM), both intracellular and secreted concentration levels of the mutant factor IX proteins were dramatically elevated to normal levels, whereas the same inhibitor concentration changed the intracellular and secreted levels of the normal factor IX only marginally by 11 and 9%, respectively.

These results indicated that mutant factor IX proteins are subjected to degradation at various efficiencies by the proteasome mechanism, whereas the normal factor IX is degraded only marginally, if at all, maintaining its maximal level of secretion. Serine protease inhibitors TPCK and TLCK at 100 μM showed no noticeable effects on the intracellular and secreted levels of either mutant or normal factor IX proteins.

DISCUSSION

A significant number of hemophilia B cases shows a unique class of phenotype, displaying nondetectable or variously reduced plasma factor IX levels with equally reduced plasma factor IX activity levels (Giannelli et al., 1994, 1996). A group of hemophilia B caused by various mutations in the C-terminal region of factor IX typically shows this phenotype. In order to gain insights into the molecular mechanisms underlying this class of mutation, we have generated various single-base mutations in the C-terminal region encompassing residues 403−415 and analyzed their effects on factor IX biosynthesis and secretion using HepG2 cells as an assay system.

Mutant factor IX proteins with various mutations in the C-terminal region showed widely varied phenotypes ranging from almost normal to nondetectable levels of secreted factor IX, while the intracellular factor IX only varied from almost normal to substantially, but not severely, reduced levels (Table 2). Presence of intracellular factor IX for the normal as well as mutant representatives are also confirmed by indirect immunostaining of the permeabilized cells (data not shown).

As shown in Figure 3 for a set of representative factor IX mutants, the reduced levels of intracellular or secreted factor IX are not accompanied by any significant changes in the factor IX mRNA levels in comparison to the normal factor IX. This indicates that the region of the factor IX gene encoding for the C-terminal region is neither involved in the transcriptional regulation of the factor IX gene nor the maintenance of the factor IX mRNA stability. Furthermore, very high intracellular levels (41−86% of the normal) in comparison to the little or nondetectable secreted levels observed for the mutant factor IX proteins indicate that the mutations in the C-terminal region are also not affecting the translational step. The specific activities observed for secreted mutant factor IX proteins were also very similar to the normal (Table 2), indicating that the C-terminal region is not involved in the tenase complex formation, which is required for factor X activation or in the activation of factor IX itself. Furthermore, the stability of factor IX mutants secreted into the culture medium are similar to that of the normal factor IX. These observations strongly suggested that the mutations in the C-terminal region are affecting the intracellular stability of factor IX proteins in the course of their secretion.

Greatly different patterns of the intracellular and secreted factor IX levels were observed with the mutations at specific amino acid residues in the C-terminal region. This suggests that these amino acid residues have varied importance in the mechanisms responsible for intracellular factor IX trafficking. These residues may be involved in the direct interactions with a chaperone protein(s) at various stages of the factor IX secretory pathway. Some mutations may cause substantial disruption of normal interactions with a chaperone protein(s) leading to efficient elimination of the mutant proteins by cellular quality control mechanisms. This hypothesis is strongly supported by the dramatic elevations of intracellular and secreted factor IX levels in the presence of proteasome inhibitors (Table 3). Proteasome has been implicated for its major role in the intracellular protein catabolism associated with the quality control mechanism of the protein trafficking pathway (Rock et al., 1994; Fra & Sitia, 1993; Pelham, 1989; Hurtley & Helenius, 1989; Tokunaga et al., 1995). In the presence of a lower concentration of the inhibitors (50 μM), the intracellular mutant factor IX levels were effectively elevated, but the secreted levels of mutant factor IX were only marginally

elevated, if any. With a higher inhibitor concentration (100 μM), however, both intracellular and secreted factor IX levels were elevated to the normal levels. These observations suggest that the major bottle neck in the mutant factor IX secretion may be in the late stage of protein trafficking, causing a significant intracellular accumulation of mutant factor IX even at a low inhibitor concentration (50 μM). The high inhibitor concentration (100 μM) apparently inhibits all the proteolytic activities, thus permitting an efficient secretion of mutant proteins. Observations on group II and IV mutants in the absence of inhibitors (Table 2), which show asymmetrically high intracellular levels compared to the much lowered secreted factor IX levels, also support this speculation. These results suggest that the mutant factor IX degradation is not very active in the early stage, but is more active in the later stage of the factor IX secretory pathway. Alternatively, sensitivity of mutant factor IX proteins to proteolytic degradation at the early stage may be different from the late stage due to substantial differences in the inhibitor accessibility to the proteasome system at different stages of the protein secretory pathway. Delineation of the specific stages of the protein secretory pathway that are particularly critical for recognition mutant factor IX proteins has yet to be made. Only the negligible inhibitor effects on the normal factor IX secretion indicate that the normal factor IX has the maximal secretory efficiency and the tight selective recognition of mutant proteins by the quality control mechanism involved in protein trafficking. Serine protease inhibitors did not significantly affect both intracellular and secreted levels of factor IX mutants.

Observations with group I and II mutations suggest that the carboxyl terminal residue T415 has a great tolerance being substituted with other amino acid residues even with leucine residue. Substitution of this residue with glycine, however, still yields substantial negative effects suggesting the important role of this threonine residue in the factor IX secretory mechanism. Moderately low levels of both intracellular and secreted factor IX in group III (R403Q, R403W, and T412S) suggest that the substantial effects of these mutations on factor IX secretion, mainly on the early and/or middle stages, but less significant effects on the late stage of the secretory pathway. It is interesting to note that a nonconservative mutation R403W did not severely impair the secretion in spite of its expected disruption of the amphipathic α-helical structure of the C-terminal region. Group IV mutations (Y404H, Y404P, W407R, I408N, T412K, and T412N) provide particularly interesting insights into the role of these residues in the secretory pathway. They apparently confer severe impairment of the late stage of the secretory pathway. The severe effects of Y404P suggest the importance of maintaining the overall conformation of the α-helical structure of this region. It is noteworthy that group IV mutations are restricted to residues Y404, W407, I408, and T412, among those tested. Severe phenotypes observed for hemophilia B patients with K411Stop and W407Stop, which may produce factor IXs with prematurely truncated C-terminal region, further support the importance of the carboxyl terminal region (Giannelli et al., 1994). Although not included in the present study, a natural mutation, V405F, found in a patient showed a plasma factor IX level of 26% with the activity level of 23%, which falls into the group II (Table 2).

Drastically reduced levels of secreted factor IX with T412K or T412N and the moderately reduced level for T412S, while all three show only moderately lower the intracellular levels, indicate the important role of the hydroxyl group of the serine side chain. Since T412S still lowered factor IX secretion by about 50%, the serine side chain apparently cannot substitute fully the function of the threonine side chain. This set of mutations (T412K, T412N, and T412S) may serve as an excellent model system for further studies into the molecular mechanisms responsible for intracellular factor IX trafficking.

Other factor IX mutations conferring the similar hemophilia B phenotype include those in the region aa 191−198 as well as a large number of other randomly distributed mutations, such as nonsense mutations and mutations at splicing sites, in addition to some missense mutations such as C56S, Y259C, and T296M. These mutant factor IX proteins may also be subjected to accelerated intracellular degradation. This, however, remains to be determined.

Enhanced intracellular clearance of abnormal proteins is also known for a significant number of other proteins including protein C and prothrombin (Halban & Irminger, 1994; Grinnell et al., 1991; Sugahara et al., 1994; Watzke et al., 1991; Yamamoto et al., 1992; Tokunaga et al., 1995). Interestingly, warfarin-treated cells do not accumulate precursor prothrombin in the ER, whereas warfarin treatment caused ER membrane associated accumulation of factor X precursor (Stanton & Wallin, 1992).

Importantly, the phenotypes (secreted factor IX levels) observed in the present study for the naturally occurring mutations, R403Q, R403W, Y404H, W407R, and T412K, reflect surprisingly well the phenotypes of hemophilia B patients with these mutations. Together with our previous studies (Hirosawa et al., 1990; Kurachi et al., 1994, 1995; Salier et al., 1990; Kurachi & Kurachi, 1995), this strongly supports the contention that the HepG2 cells can be utilized as a durable assay system for studying factor IX biosynthesis.

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