The effects of novel mutations in A1 domain of human coagulation factor VIII on its secretion level in cultured mammalian cells

Gholam Ali Kardar[1,2], Alireza Zomorodipour[1*], Mostafa Moin[2], Zahra Pourpak[2], Mehdi Sadeghi[1], Fariba Ataei[1]

[1]National Institute of Genetic Engineering and Biotechnology, P.O. Box 14965/161, Tehran, I.R. Iran
[2]Immunology, Asthma and Allergy Research Institute, Children's Medical Center Hospital, Tehran University of Medical Sciences, P.O. Box 14185-863, Tehran, I.R. Iran

Abstract
Inefficient secretion of the human coagulation factor (hFVIII) in mammalian expression systems is one of the main causes of the *hFVIII* low expression level, attributed to its interaction with a chaperone known as BiP/GRP78. In order to improve secretion efficiency of the hFVIII, based on the higher secretion level of the porcine FVIII and analysis of the hFVIII A110 region, that inhibits its secretion, function of three novel B-domain deleted hFVIII mutant including; two single-mutants (Leu299Phe and Phe309Thr) and a double-mutant (Tyr323His/Lys325Arg) were examined in three mammalian cell lines (HEK-293T, COS, CHO) for the hFVIII secretion efficiency. The double-mutant construct displayed the highest hFVIII expression level, about seven-fold as much the base-line. The double-mutant hFVIII was biologically active and its inactivation patterns by EDTA and heat was similar to that of the non-mutant hFVIII. Semi-quantitative RT-PCR results showed the highest mRNA level for the double-mutant *hFVIII*. Both of the mutated residues in the double-mutant are located in a hydrophobic heptamer (^{320}MEAYVKV^{326}) that seems to be involved in Bip-binding activity. None of the L299F and F309T hFVIII mutants exhibited improved secretion. This result has provided convincing evidence for the increasing effect of the double-mutant on the hFVIII secretion and transcription efficiencies.
***Keywords*:** Hemophilia A; human coagulation Factor VIII; BiP; A110 region; Secretion; mammalian expression system

Correspondence to:* **Alireza Zomorodipour, Ph.D.
Tel: +98 21 44580348; Fax: +98 21 44580906
E-mail: zomorodi@nigeb.ac.ir

INTRODUCTION

Human coagulation factor VIII (hFVIII) is a large glycoprotein that participates in the intrinsic pathway of the coagulation cascade as a cofactor of factor IX in the proteolytic activation of factor X (Mann, 1999). The hFVIII is synthesized as a large single-chain multi-domain glycoprotein with the structural domains of A1-A2-B-A3-C1-C2 (Lenting *et al*., 1998; Vehar *et al*., 1984). The B-domain is not required for procoagulant activity and released proteolytically upon activation by thrombin (Pittman *et al*., 1993). Absence and malfunctioning of FVIII lead to the bleeding disorder hemophilia A, the most commonly inherited bleeding disorder, affecting approximately 1-2 in 10000 males (Antonarakis *et al*., 1995). The current treatment for hemophilia A is replacement therapy, being done through the intravenous infusion of either plasma-derived or recombinant forms of the hFVIII (Hoeben *et al*., 1995; Rosendaal *et al*., 1991). However, concern over the possible transmission of blood-borne pathogens such as *hepatitis viruses*, *HIV* and *parvovirus B19* in addition to prions in the plasma derived products, has persuaded many researchers to pay special attentions over the production of recombinant hFVIII (Cervenakova *et al*., 2002; Peerlinck and Vermylen, 1993; Gerritzen *et al*., 1992) . To produce a biologically active FVIII, a mammalian expression system is required to provide proper post-translation modifications. However, expression of the full-length of the *hFVIII* in heterologous mammalian systems is much less efficient compared with other proteins of similar size (Miao *et al*., 2004). Some reasons have been iden-

tified that limit the hFVIII expression, including those involved in transcription and post-transcription processes (Kaufman *et al*., 1989). In this regard, reduced accumulation of FVIII mRNA has been reported in various cell types, such as Swiss 3T3, primary human skin fibroblasts, Rat2 fibroblasts, and Chinese hamster ovary (CHO) cell lines (Hoeben *et al*., 1995; Lynch *et al*., 1993; Kaufman *et al*., 1988). Inefficiencies in intracellular transport and secretion of FVIII and sensitivity of this protein to proteolytic degradation were also described as the post-transcription processes, which cause the low expression and secretion levels of the hFVIII in mammalian expression systems (Kaufman *et al*., 1997; Pittman *et al*., 1993; Dorner and Kaufman, 1990; Kaufman *et al*., 1989; Kaufman *et al*., 1988).

The hFVIII gene contains sequences that dominantly inhibit its own expression (Mannucci and Tuddenham, 2001; Hoeben *et al*., 1995). A 31-bp fragment corresponding to nt 1569 to 1600 of the FVIII cDNA, interacts with factors present in nuclear extracts and represses the gene expression (Fallaux *et al*., 1996). Further evidences for the presence of inhibitory elements within the coding region of hFVIII was provided from the study of a hFVIII/FV chimeric protein which demonstrated that sequences within the C-terminal of the A1 domain in FVIII inhibit secretion of eukaryotic cells (Marquette *et al*., 1995). Another evidence for the presence of inhibitory element within the A1 domain of the hFVIII has been provided from the fact that the porcine FVIII (pFVIII), as the hFVIII orthologue, is expressed 10-14 fold higher than the hFVIII (Doering *et al*., 2002). They even showed an increased secretion level for a porcine/human hybrid FVIII in comparison with that of the hFVIII (Doering *et al*., 2004; Hay, 2002). Inefficient secretion of the hFVIII protein, which is one of the main causes of its low expression level, has been attributed to its interaction with a chaperone known as BiP/GRP78 (Dorner *et al*., 1992). A majority of newly synthesized FVIII proteins in ER lumen interact with BiP through a 110 amino acid region within the A1 domain, known as A110 region. Accumulation of unfolded proteins in ER lumen results in the ER response by activating a number of intracellular signal transduction pathways, known as unfolded protein response (UPR) (Pipe *et al*., 1998). Because of this response, a transient reduction in the protein load in ER occurs by the lower protein synthesis and translocation into the ER and the higher capacity of ER to handle the unfolded proteins (Ron and Walter, 2007; Pipe *et al*., 1998). The human Factor V (hFV) and hFVIII both have six similar structural domains (A1-A2-B-A3-C1-C2) with approximately 40% amino acid sequence homology in the A and C domains, but the hFVIII secretion is seven fold less efficient than that of the hFV (Pipe, 2009; Miao *et al*., 2004; Marquette *et al*., 1995; Pittman *et al*., 1994). The higher secretion level of the hFV might therefore be due to the fact that it does not bind to BiP (Nichols and Ginsburg, 1999; Pipe *et al*., 1998). Roles of other ER lectin chaperones such as calnexin, calreticulin and LMAN1 in this process have also been highlighted (Cunningham *et al*., 2003; Moussalli *et al*., 1999; Pipe *et al*., 1998; Kaufman *et al*., 1997) . It was shown that peptides containing heptamers, enriched in large hydrophobic and aromatic amino acids with the Hy (W/X) HyXHyXHy sequence bind to BiP with high affinity and stimulate the ATPase activity of BiP. In this motif "X" stands for any amino acid, "Hy" stands for either large hydrophobic or aromatic amino acids and "W" stands for Tryptophan (Blond-Elguindi *et al*., 1993). Altogether, the data mentioned above strongly suggest that the A110 amino acid fragment within the A1 domain has inhibitory effect on secretion of the hFVIII. So, one approach for enhancement of FVIII secretion would be to reduce binding of BiP chaperon to FVIII (Nichols and Ginsburg, 1999; Pipe *et al*., 1998). In this regard, Swaroop *et al*. showed that two mutations L303E and F309S in A1 domain of FVIII (BiP binding site) dramatically improve secretion of FVIII into culture medium more than three fold (Swaroop *et al*., 1997).

In the present work, based on the bioinformatic analysis of the hFVIII protein sequence, four novel amino acid substitutions were introduced into the A1 domain of a B-domain deleted hFVIII (BDD-hFVIII) using site-directed mutagenesis and subsequently, the functions of the introduced mutations on the hFVIII expression were studied in several mammalian cell lines.

MATERIAIS AND METHODS

Desk-top analysis: The human and porcine FVIII protein sequences were obtained from gene bank (NCBI) with the accession numbers of CAI41672 and NP-999332, respectively. Sequence similarity search was performed using EBI/Blast2p (Altschul *et al*., 1997). The amino acid residues on the protein surface were evaluated based on the solvent accessible surface area of each amino acid residue using the GetArea program

(http://curie.utmb.edu/getarea.html).

Bacterial strains, mammalian cell lines, plasmids, and primers: The *DH5α* (Strategene, USA) strain of *Escherichia coli* was used for cloning and sub-cloning steps. The CHO and COS-7 cells (National Cell Bank of Iran, Pasteur Institute of Iran) and Hek293T cell line (ATCC, USA) kindly provided by Dr Gardaneh; NIGEB, were used as the mammalian expression hosts. Plasmid pcDNA3-hFVIII, reported previously (Amirizadeh N *et al*., 2005), was used as a source of the B-domain-less *hFVIII* cDNA. Primers used for the PCR-mediated site directed mutagenesis are listed in Table 1 (synthesized by MWG, Germany).

Media, enzymes, chemicals and kits: Luria-Bertani (LB) was used as a culture medium for bacterial growth. Ampicillin (100 µg/ml) was added to the culture medium when required to maintain selection pressure. Enzymes including *Kpn*I, *Mun*I, *BamH*I, *Pfu* and *Taq* DNA polymerase and *T4* DNA ligase and other chemicals such as FuGene-6 and geneticin (G-418) were purchased from Roche, Germany. Commercially prepared columns (Roche) were applied for purification of plasmids and DNA fragments from agarose gel as well as PCR product. The CHO cells were grown in Dulbeco's modified Eagle's medium (DMEM) and Hams-F12 (Gibco, Germany) with 1 to 1 ratio, Cos-7 and Hek293T were cultured in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco) in addition to 100U/mL penicillin G and 100µg/mL streptomycin (Sigma, USA). During stable expression analysis, the culture medium was supplemented with 1% MEM non-essential amino acids (Sigma). A PCR-product cloning Kit (InsT/Aclon Fermentas, Russia) was used for the cloning of the PCR products. ELISA kit for the measurement of the hFVIII antigen and deficient FVIII plasma were purchased from Diagnostica Stago-France. Citrated normal pooled plasma (kindly provided by Dr Amirizadeh in the quality control unit of the Iranian Blood Transfusion Organization) was used as a standard sample in the coagulation test. FVIII activity was determined by the COATEST FVIII kit (Chromogenix Inc., Italy).

Site directed mutagenesis of the hFVIII: To study the effects of four amino acid substitutions at positions 299 (Leu→Phe), 309 (Phe→Thr) and 323 (Tyr→His)/325(Lys→Arg) in the A1 domain of the hFVIII, the codons corresponding to those locations were changed in a CMV-regulated expression plasmid carrying a BDD-hFVIII (Amirizadeh N *et al*., 2005) in three different experimental lines. To do this, three independent Splice Overlap Eextension-PCR (SOE-PCR) were carried out (Hoeben *et al*., 1990). Oligonucleotides f8bam-F and f8bam-R were used as the common external primers with *BamH*I sites on their 5' ends and other 6 oligonucleotides matching the mutation sites were applied as three pairs of internal overlapping primers (Table 1). For each construct in the first step, two PCRs corresponding to two overlapping fragments were carried out and their products were extended with a 3rd PCR reaction, to generate a 2kb mutated sub-fragment of the *hFVIII* cDNA. The first two PCRs in each SOE-PCR were carried out based on the specific temperature profile designed based on their corresponding oligonucleotides sequences. The 3rd PCR in each case, composed of 5 cycles without the external primers, followed by 25 cycles with the external primers with the following temperature profile: after an initial denaturation step at 94°C for 4 minutes the program continued for 30 cycles of 94°C for 45 second, 68°C for 45 second, 72°C for 2 minutes, followed by a final extension at 72°C for 7 minutes.

Construction of recombinant plasmids: The three hFVIII-derived fragments, equipped with *BamH*I sites on both ends, were used to substitute their wild type counterparts in the pcDNA3-hFVIII plasmid (pW), after *BamH*I digestions. The recombinant plasmids were transferred into the *DH5α* strain of *E. coli* that were subsequently isolated on ampicillin containing media. Orientation of the inserted *BamH*I/*BamH*I fragments in the expression plasmids were determined by restriction analysis (not shown). The introduced mutations were confirmed by complete nucleotide sequencing of both strands of the cloned fragments using ABI 373A automated sequencer (MWG). The experiments resulted in construction of three *hFVIII* expressing plasmids; pLF (L299F), pFT (F309T) and pYH/KR (Y323H/K325R), which were used for expression analysis in parallel with their parental plasmid carrying a BDD-hFVIII (pW) (Amirizadeh *et al*., 2005).

Transfection and expression analysis: To achieve stable hFVIII expressing cell lines the CHO cell line was considered as host. With this aim, about 2.5×10^6 CHO cells were transfected with 15 µg of the *Mun*I-digested (linearized) recombinant plasmids using a Gene Pulser Xcell electroporation system (BioRad,

USA) and cultured at 37ºC in 5% CO_2 humidified conditions for 48 hours. After selection with 550 µg/ml G418 in FBS containing media, resistant colonies of each transfection line were pooled for further expression analysis in comparison with the CHO cells transfected with a parental pW plasmid. The pooled cells were seeded at 4×10^5 cells/2 ml/well in 6-well plates and their corresponding conditioned media were collected every 24 hours for three days.

For transient expressions 3 µg of circular DNA of the recombinant plasmid was used to transfect the COS-7 and Hek293T cells with FuGen6 according to the manufacturer's instructions (Roche). The tansfection experiments were carried out in triplicates and conditioned medium was harvested every 24 h during three days after transfections.

Measurement of the hFVIII antigene (hFVIII:Ag): The recombinant hFVIII (rhFVIII) antigens both in the conditioned cultured media and within the expressing cells were assayed by ELISA using a microplate coated with a specific anti-hFVIII antibody, provided in the kit (Diagnostica Stago Co., France). A standard curve was drawn from 2-fold serial dilutions of lyophilized human normal pooled plasma, containing a FVIII level at 1000 mIU/ml and the concentration of the hFVIII in culture medium was expressed as mIU/ml.

Assessment of intracellular accumulation of the hFVIII: In order to examine the intracellular accumulation of the expressed hFVIII levels, the transfected cell extracts were prepared in ice-cold lysis buffer containing: 0.05 M Tris-HCl pH 8.0, 0.15 M NaC1, 1% (w/v) NP-40 and protease inhibitor cocktail (Roche), using three cycles of freeze-thaw lysis (David *et al*., 2001). The cell lysate was centrifuged at 12,000 g for 15 min at 4°C and the supernatant was used for analysis. Concentration of total protein of the lysate was measured by Bradford assay (Bradford, 1976). The hFVIII antigen (hFVIII:Ag) in cell lysate was measured by ELISA and normalized to the total cell-protein of the cell lysate determined based on Bradford method and finally stated as ng/mg protein.

hFVIII activity assay (hFVIII:C): The biological activity of the secreted hFVIII was measured using the COATEST FVIII kit (Chromogenix Inc., Italy) according to the instructions described by manufacturer. Normal human pooled plasma was considered as standard with 100% activity equal with 1 IU of hFVIII per ml. A single-step coagulation test was carried out to perform inactivation test using immuno-depleted plasma for FVIII and activated partial thromboplastin (aPTT) reagent (Diagnostica Stago, France) according to the manufacturer's instructions.

hFVIII inactivation test: Inactivation of the hFVIII in the cultured media was carried out either by heating the samples at 50°C or EDTA (10 mM) treatment at room temperature. Subsequently, the hFVIII activities of the samples were examined after various inactivation time using a one-stage clotting assay (Swaroop *et al*., 1997).

RNA Extraction and Semi-quantitative RT-PCR: Total RNA was extracted from cells using Tripure RNA-isolation reagent according to the manufacturer's instruction (Roche). First-strand cDNA was syn-

Table 1. Primers used for the mutagenesis of the BDD-hFVIII cDNA.

Primers	Primer sequences (5'➔3')
f8bam-F	CGCGGATCCACTAGTAACGGCCGCCAGTGT
f8bam-R	TCTGGATCCTCAAGCTGCACTCCAGCTGGA
f8F309T-F	CTACTGACATGTCATATCTCTTCCCAC
f8F309T-R	AGAGATATGACATGTCAGTAGAAACTG
f8L299F-F	AAACATTTTTGATGGACCTTGGACAG
f8L299F-R	TCCAAGGTCCATCAAAAATGTTTGAGC
f8YHKR-F	ATGGAAGCTCATGTCAGAGTAGACAGC
f8YHKR-R	ACAGCTGTCTACTCTGACATGAGCTTC
β-actinF	GAGACCTTCAACACCCCAGCC
β-actinR	AGACGCAGGATGGCATGGG
A1-8F	TTAAGAATGCGGCCGCATGCAAATAGAGCTCTCCAC
A1-8R	AGTCCTGAAGCTAGATCTCTCTCC

thesized using 1 µg total RNA in 40 µl reaction mixture consisting of 8 µl 5X reaction buffer, 2 µl 10 mM dNTPs (Roche), 2 µl 10 Pmol/ml random hexamer (Roche), 2 µl DTT 0.1 M, 1 µl RNase inhibitor (Roche) and 2 µl Moloney Murine Leukemia Virus (M-Mulv) reverse transcriptase (200 u/ml) (Fermentas). The mixture was incubated at 37°C for 60 min, followed by 10 minutes incubation at 70°C. Primers A1-8F and A1-8R were used to amplify a 1700 bp fragment of the *hFVIII* transcript. For internal control, specific primers were used to amplify a 180 bp fragment of the human *β-actin* gene for normalizing the expression values (Table 1).

Statistical analysis: Every one of the expression analysis assays including coagulation test and ELISA, were carried out in triplicates and the generated data were presented as the means ± SD. The Mann-Whitney U test was used to evaluate differences among the mini-genes. A P value of less than 0.05 was considered as significant difference. All statistical analyses were carried out with SPSS 11.5 (SPSS Inc.).

RESULTS

Analysis of the hFVIII A110 region: Bearing in mind the postulated interaction of the A110 region within the hFVIII A1 domain with BiP and therefore its function on the secretion efficiency of the hFVIII (Marquette *et al*., 1995), a bioinformatic analysis was carried out to locate some of residues that are more likely involved in interaction with BiP (Fig. 1). With this aim and based on different evidences, reported previously, four target sites in the hFVIII A110 region were selected for subsequent bioengineering of the hFVIII, as described in the following.

In our first plan for mutagenesis, the proposed function of the hydrophobic heptamers in binding of the proteins to BiP was considered (Blond-Elguindi *et al*., 1993), and the amino acid sequence of the hFVIII A110 region was screened for such potential hydrophobic heptamers. In total, 10 hydrophobic heptamers were found, among them only one, ^{320}MEAYVKV^{326}, matches with the suggested motif (Fig. 1A). Interestingly, two residues in this heptamer including Y^{323} and K^{325} are among those 13 amino acids, which are different from that of the pig FVIII (pFVIII). Therefore, these two residues were also subjected for amino acid substitutions with their counterparts in the pFVIII Y323H and K325R in the first mutant (Accession no. FN811132).

For the next mutation design, the higher secretion level of the pFVIII in comparison with the human FVIII was considered. Out of the 13 different amino acids between their A110 domains (Doering *et al*.,

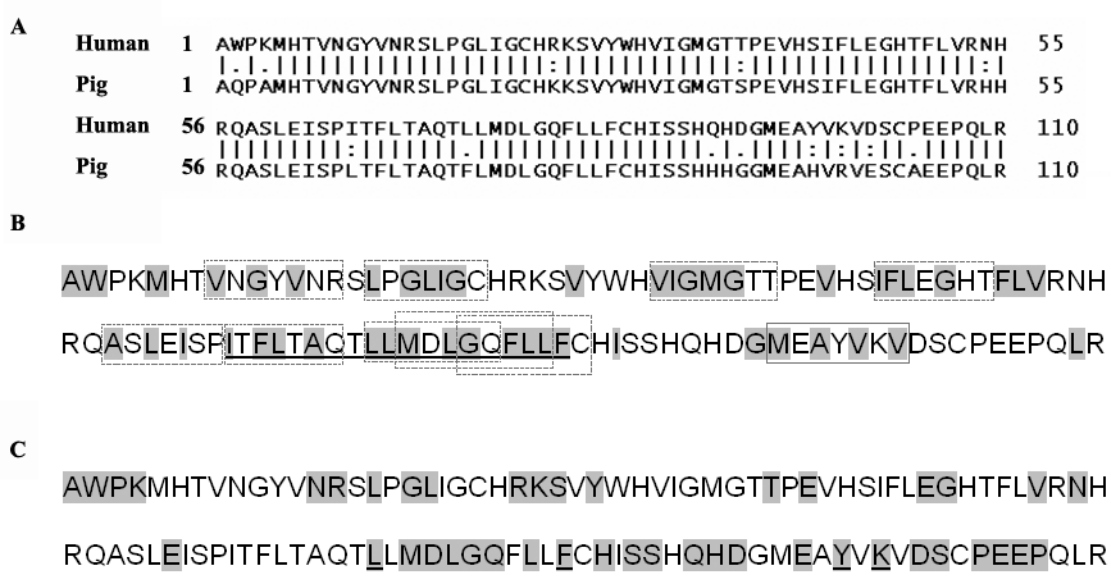

Figure 1. Desk-top analysis of the amino acid sequence of the hFVIII A110 region. A; Pair wise alignment of amino acid sequences of the A110 regions of hFVIII and pFVIII. Identity and similarity of the amino acid sequences are 88.2% and 94.5% respectively. B; The Hydrophobic amino acids within the hFVIII A110 region are highlighted. Underlined amino acid sequence has been reported as a candidate for BiP interaction (19). The hydrophobic heptamers are boxed. C; Highlighted residues in the hFVIII A110 region with side chain surface area values greater than 30%. The underlined amino acids have been mutated in this study.

2004; Swaroop *et al*., 1997), only one (L299F) is present in the area (I^{291}-F^{309}), where previously was suggested as the major BiP-binding site (Marquette *et al*., 1995) (Fig. 1). So, this point was considered the next point for a L299F substitution mutagenesis, in the second mutant.

Swaroop and colleague (1997) showed that substitution of F309S as a non-polar amino acid increases the hFVIII secretion (Swaroop *et al*., 1997). Based on their achievements, in our last mutation design, threonine as another member of the non-polar amino acids was considered to substitute the Phe^{309}.

Considering the importance of the surface amino acids in protein-protein interactions and role of their corresponding side chains in this regard, the A110 region was analyzed using the hFVIII PDB data (http://www.rcsb.org/pdb/home/home.do) and the GetArea program and 43 residues with the side chain surface area value greater than 30% were detected. All the four target sites for mutagenesis in this study appeared to be in this group (Fig. 1).

Based on the four mutations mentioned above, three mutated *hFVIII* expressing plasmids; pLF, pFT and pYH/KR were constructed having CMV-regulated BDD-hFVIII expressing plasmid as the parental plasmid.

Expression analysis of the hFVIII mutants: The recombinant plasmids in parallel with the parental BDD-hFVIII expressing plasmid (pW) were used to transfect three different cell lines; CHO, Cos7 and Hek293T. In the case of CHO cells, permanent expression of the hFVIII was aimed. In other two cell lines; Cos7 and Hek293T which stably express the large T-antigen of SV40 and cause the effective replication of episomal plasmids in the lines (Gluzman, 1981), transient expressions of the hFVIII were aimed. Therefore, linear forms of the four recombinant plasmids were used to transfect the CHO cells and their circular forms were used for the transfection of the other two cell lines.

As the results obtained from the hFVIII:Ag measurements of the cultured media indicate, secretion levels of FVIII by the pYH/KR construct in all the examined cell lines were about seven fold as much the baseline. In the cases of the pLF or the pFT mutants, the hFVIII secretion levels were below the base line (Fig. 2). The increased secretion level of the hFVIII by the pYH/KR mutant in comparison with those of the wild type and other mutants was also evidenced from the coagulation activities of their corresponding samples

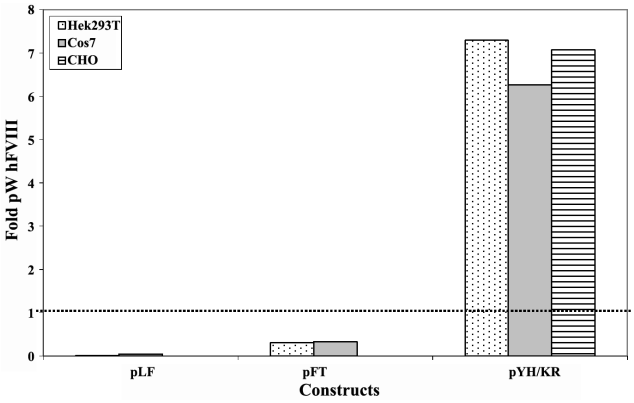

Figure 2. Secretion of the hFVIII by three cell lines (Hek293T, Cos7, CHO) transfected with three mutant hFVIII expressing plasmids as indicated by the hFVIII:Ag measurement compared with the hFVIII secreted from the cells transfected by pW. Base line is demonstrated by values obtained from the pW containing cells. pFT: F309T., pLF: L299F., pYH/KR: Y323H/L325R.

(Table 2). As the data obtained from the measurements of hFVIII expression indicates among the examined cell lines, the Hek293T cells appeared as the most efficient host for the expression of the hFVIII mutants.

Assessment of the intracellular hFVIII: One way to evaluate the assumed effects of the introduced mutations on the secretion of the expressed hFVIII, was to assess the accumulation of the hFVIII inside the recombinant cells. As the most efficient recombinant hFVIII expressing cells, the Hek293T cells were subjected for the assessment of the intracellular hFVIII. With this aim, the cell lysates corresponding to the Hek293T cells which were transfected with the four different plasmids were subjected for the measurement of the hFVIII antigenicity and normalized to total protein levels (Fig. 3). With the exception of the pFT containing cells, the intracellular hFVIII contents of the examined cells more or less follow those of their corresponding secreted hFVIIIs. In this context, the highest and the lowest levels of the hFVIII secretions by the pYH/KR and pLF related clones are followed by their highest and lowest intracellular hFVIII contents, respectively. Similarly, low intracellular FVIII content of the pW containing cells follows its low extracellular content of FVIII. In the case of the pFT containing cells, which showed a very low secretion level of FVIII, the expressed hFVIII was considerably accumulated inside the cells. This is even comparable to that of the intracellular hFVIII in the pYH/KR-con-

Table 2. The hFVIII expression analysis of the cultured media from three cell lines (Hek293T, Cos7 & CHO) transfected with three mutant hFVIII expressing plasmids based on the hFVIII:Ag and hFVIII:C measurements (Mean±SD). The Values are stated in mIU/106cell/mL/day.

Constructs	Hek293T		Cos-7		CHO	
	Antigen	Activity	Antigen	Activity	Antigen	Activity
pW	47.44 ± 7.7	30.42 ± 9	5.29 ± 2.8	1.96 ± 1.8	2.11 ± 0.8	1.28 ± 0.7
pLF	0.58 ± 0.6	1.34 ± 1.1	0.25 ± 0.3	0.62 ± 0.8	0	0.22 ± 0.4
pFT	14.73 ± 4.3	1.9 ± 2.3	1.77 ± 1.2	0.23 ± 0.4	0	0
pYH/KR	345.99 ± 17.6	110.69 ± 35.5	33.13 ± 6.7	13.8 ± 4.2	14.9 ± 1.9	7.49 ± 0.8

taining cells that showed the highest content of both intracellular and extracellular hFVIII among the examined cells.

Inactivation patterns of the expressed hFVIII: Biological activity of the hFVIII secreted into the conditioned media by the hek293T cells containing the pYH/KR plasmids in comparison with that of the pW containing Hek293T cells was evaluated by performing two separate inactivation tests; either heating the samples at 50°C or treating them with 10 mM EDTA for various durations (Fig. 4). The results obtained from both of the inactivation tests demonstrate a similar inactivation patterns for the proteins expressed from the pW and pYH/KR construct.

Evaluation of the hFVIII transcription: A comparative expression analysis at transcription level was performed among the Hek293T cells transfected with plasmids pW, pLF, pFT and pYH/KR. With this aim, mRNAs isolated from the four different recombinant Hek293T cells were subjected for a semi-quantitative RT-PCR analysis using specific primers. For normalization, PCR with *β-actin*-specific primers was performed based on the method described by VandenDriessche *et al*. (VandenDriessche *et al*., 1999). As expected, endogenous *β-actin* mRNA could be detected by RT-PCR in all samples as well as the negative control. The *hFVIII* mRNA was detectable in all the examined cells, not in untransfected cells. No significant differences were observed between the *hFVIII* transcription level of the cells transfected with the pW plasmid and those transfected with plasmids pLF and pFT. However, a relatively higher transcription level for the pYH/KR containing cells in comparison with other examined cells was evidenced. This result is in consistent with the results obtained from ELISA (Fig. 5).

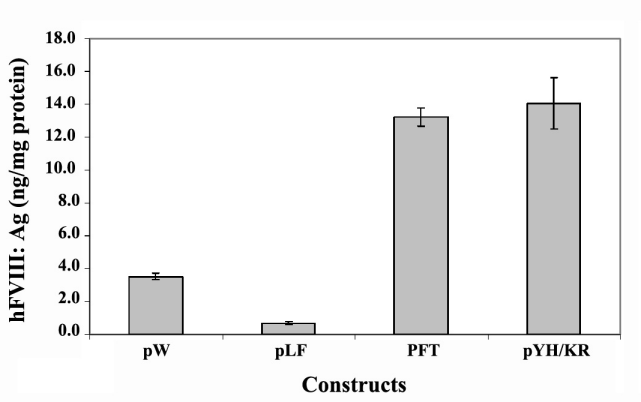

Figure 3. Estimation of the intracellular hFVIII of the Hek293T cells transfected with different mutated hFVIII expressing plasmids, based on FVIII:Ag (mIU/mg total protein), using ELISA. Detectable level of hFVIII was not identified in lysate of non-transfected cell. The results represent the mean values of the intracellular hFVIII of three independent experiments. The mutants corresponding names are indicated below the columns. pFT: F309T., pLF: L299F., pYH/KR: Y323H/L325R.

DISCUSSION

In our previous study, expression of a BDD-hFVIII in CHO cells was shown (Amirizadeh N *et al*., 2005). The highest hFVIII expression level obtained from the stably transfected CHO cells was 0.1 U/ml in the cultured media (Amirizadeh N *et al*., 2005). Due to results obtained by other researchers, the low expression level of hFVIII was not a surprise (Lynch *et al*., 1993). The low level of FVIII expression and increasing demands for prophylactic treatment of hemophilia A patients have increased the need for the production of rhFVIII (Hay *et al*., 1999). Various solutions have been applied to improve the hFVIII expression in cultured mammalian cells. Use of the BDD-FVIII mutant itself was to increase the mRNA yield (Pittman *et al*., 1993). Many research groups used multiple rounds of selections with methotrexate for the amplification of the expressed *hFVIII* and reported expression levels of

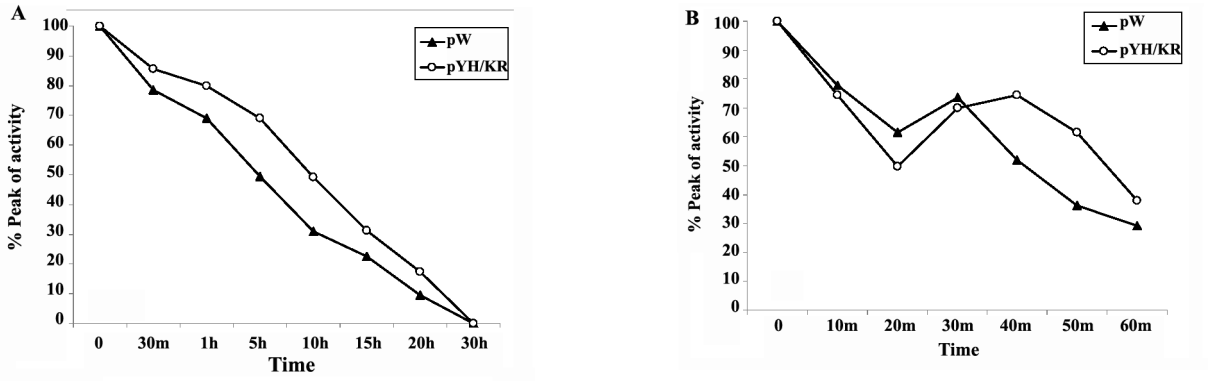

Figure 4. Inactivation pattern of the expressed hFVIII from the recombinant Hek293T transfected with the pW and pYH/KR constructs, by either heating the samples at 50oC A: or EDTA treatment B: for various durations. m: minute; h: hours. pYH/KR: Y323H/L325R.

0.2-1 U/ml hFVIII in dHFR- CHO cells (Soukharev *et al*., 2002). Inefficient secretion of the hFVIII protein, which is one of the main causes of its low expression level, has been attributed to its interaction with a chaperone known as BiP/GRP78 (Dorner *et al*., 1992). Role of the A110 region within the carboxyl-terminal half of the hFVIII A1 domain to inhibit the secretion of FVIII from mammalian cells by retaining it in the ER or altering protein folding has been previously reported (Pipe *et al*., 1998; Ron and Walter, 2007). Within this region a hydrophobic cluster of residues from I^{291}-F^{309} contains multiple 7-mer peptides having a high probability of binding BiP (Marquette *et al*., 1995). In this regard, changing of an amino acid (F309S) was shown to improve the hFVIII secretion (Swaroop *et al*., 1997). Accordingly, it was suggested that a primary BiP-binding site likely resides in the FVIII A1 domain. This was also confirmed later by Miao *et al*. who showed that incorporation of the point mutation (F309S) within a putative BiP binding region of the A1-domain increases the hFVIII secretion (Miao *et al*., 2004). This improvement occurred probably as a result of either a weaker interaction of the mutant protein to BiP or improved folding properties of the expressed hFVIII (Swaroop *et al*., 1997). Some other groups have also tried to improve the hFVIII secretion deficiency through the bioengineering of the hFVIII (Saenko and Pipe, 2006; Fay and Jenkins, 2005; Pipe, 2005).

In the present study, we have targeted four positions in the A110 region of the A1-domain of the hFVIII for amino acid substitutions. These positions are thought to play important roles in binding of the protein to BiP and it was assumed that their substitutions with suitable amino acids could improve the secretion efficiency of the hFVIII. Among the examined mutants the one with two mutations, pYH/KR, displayed the highest FVIII expression level in all the three examined cell lines. Secretion levels of the hFVIII by the pYH/KR construct in all the three exam-

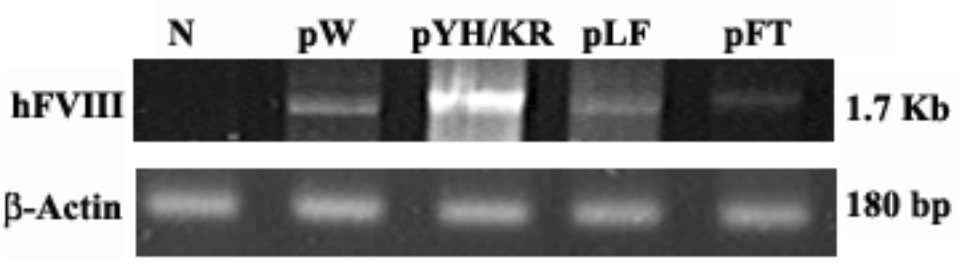

Figure 5. PCR products amplified from the reverse transcribed total RNA prepared from the recombinant Hek293T cells. In each case, the prepared RNA was treated with RNase-free DNase and subjected to semi-quantitative RT-PCR analysis using hFVIII specific primers (b-actinF and b-actinR). b-Actin specific primers were used as internal control. N: negative control (Untransfected Hek293T).

ined cell lines were about seven-folds as much the base-line. Among the examined cell lines, the Hek293T cells appeared as the most efficient host for the expression of the hFVIII. The mutated residues in the double mutant were designed based on the amino acids in corresponding positions in the pFVIII, due to its higher secretion efficiency (Doering *et al*., 2002). Both of these residues appeared to be located in a hydrophobic heptamer (^{320}MEAYVKV^{326}), with high affinity to BiP (Blond-Elguindi *et al*., 1993). Therefore, it suggests for a critical role of this heptamer in BiP binding of the hFVIII. Interestingly, the residues, in particular Y^{323} and K^{325}, are among those with the side-chain surface area value greater than 30% (Fig. 1C), which support their probable involvement in a sort of protein-protein interaction between the hFVIII and BiP.

According to the results obtained from the measurement of the hFVIII contents of both cultured medium and lysate of the pYH/KR containing Hek293T cells, the double-mutant exhibited the highest hFVIII expression level. Therefore, either one or a combination of the two mutations is responsible for the improved hFVIII expression, remains to be investigated. This result is comparable to the results obtained by Swaroop *et al*. who demonstrated a three-fold increase in the secretion level of a L303E/F309S hFVIII mutant (Swaroop *et al*., 1997). Based on the results provided by Swaroop *et al*., the F309S mutant increased the hFVIII secretion (Swaroop *et al*., 1997), whereas in the case of the F309T, in this study, no effect was observed. Although both Serine and Threonine are similar, they are still different in their chemical structure, in a methyl group, which may be involved in this regard. The inactivation patterns of the expressed hFVIII from the pYH/KR mutant appeared to be similar to that of the pW containing cells. In contrast, in the F309S hFVIII mutant, reported by Swaroop *et al*., the EDTA treated inactivation resulted in approximately 10-fold reduction in the mutant hFVIII biological activity in comparison with the wild type hFVIII (Swaroop *et al*., 1997).

To examine the secretion efficiencies of the mutants, in the first step, we looked in the cultured media that indicated a higher expression (secretion) level of the hFVIII for the double mutant in comparison with the wild BDD-hFVIII. However, the higher level of hFVIII in the intracellular compartments of the double mutant comparing with the wild type suggested for a probable increase in the expression of the *hFVIII* at transcription level. Therefore, we examined their corresponding mRNAs based on a semi-quantitative RT-PCR and found that the mutations (in the double-mutant) which are effective on the hFVIII secretion are also probably effective at the transcriptional level. The increased expression observed in this mutant at transcriptional level can be explained by the function of mutations either on the mRNA structure or as enhancer at transcription step.

Neither the pLF mutant nor the pFT containing cells exhibits improved secretion efficiency. However, the lower intracellular hFVIII content in the pLF containing cells compared with those of the pFT and pW can be explained by a probable function of UPR response as a result of a strong interaction of the expressed FVIII with BiP. This response has been triggered by a probable conformational change of the expressed hFVIII protein due to the introduction of the L299F mutation, into degradation process, based on Endoplasmic Reticulum Associated Protein Degradation (ERAD) mechanism (Ron and Walter, 2007). In this regard, the relatively higher intracellular content of the hFVIII in the pFT containing cells can be explained by the fact that the probable conformational change in this mutant did not lead to activation of the ERAD mechanism.

Most of the data obtained from the study of the hFVIII-Bip interaction, indicate that the A110 region and particular amino acid sequences in there play key-roles in this regard (Pipe *et al*., 1998; Ron and Walter, 2007). However, there are reports that show mutations R593C and N618S in the hFVIII-A2 domain and mutations R2307Q/l and Y2305F in the hFVIII-C2 domain result in intracellular accumulations of the hFVIII and cause a dramatic reduction in secretion efficiencies of the hFVIII in haemophilia patients (Pipe and Kaufman, 1996; Roelse *et al*., 2000). Therefore, it is suggested that the hFVIII binding to BiP is mediated not only through a primary sequence motif within the A1 domain, but also through a conformational motif. The later can include the amino acids from various domains of the hFVIII, which should be examined by performing both bioinformatic and experimental analysis.

Previous studies demonstrated that replacement of one or more domain(s) of the hFVIII with corresponding domains of the pFVIII could increase the hFVIII secretion level (Doering *et al*., 2004). This study has provided evidences that replacement of only two amino acids in the A1 domain can increase hFVIII expression at both transcription and secretion levels.

In conclusion, the potential of a double-mutant

BDD-hFVIII (pYH/KR) for an improved expression of a biologically active hFVIII in cultured mammalian cell line has been shown. However, this mutant showed a high tendency for the accumulation of the expressed hFVIII within the intracellular compartments of the cells. One of the key elements in secretion efficiencies of proteins is their attached signal peptide which must be processed efficiently (Blobel *et al*., 1979). Accordingly, one approach to improve the secretion efficiency of the hFVIII during an over expression state would be to examine the function of heterologous signal peptides. Use of a combination of the BDD-hFVIII mutants, in particular the double mutant (pYH/KR), and other modifications such as the introduction of the first 226 amino acids of B domain (Miao *et al*., 2004), application of introns (Plantier *et al*., 2001), and use of an efficient signal peptide are also alternative solutions for future studies to achieve an increased secretion of rhFVIII.

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