Intracellular retention of a factor VIII protein with an Arg²³⁰⁷ → Gln mutation as a cause of haemophilia A

Jan VOORBERG*, Rozalia T. M. DE LAAF, Pauline M. KOSTER and Jan A. VAN MOURIK

Department of Blood Coagulation, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands

Substitution of Arg²³⁰⁷ by Gln in factor VIII has been found to be associated with mild to moderate haemophilia A [Gitschier, Wood, Shuman and Lawn (1986) *Science* **232**, 1415–1416]. We have introduced this particular point mutation into a B-domain-deleted factor VIII cDNA and expressed the modified cDNA in C127 cells. Cells expressing the resulting protein, termed des-(868–1562)-factor VIII-R2307Q, were compared with those expressing the previously characterized des-(868–1562)-factor VIII. No immunoreactive material could be detected in the conditioned medium of cells transfected with des-(868–1562)-factor VIII-R2307Q cDNA using assays specific for the factor VIII light chain and the factor VIII heavy chain. Analysis of metabolically labelled cells transfected with des-(868–1562)-factor VIII-R2307Q cDNA revealed that this mutant protein is synthesized

at a level similar to des-(868–1562)-factor VIII. In contrast to des-(868–1562)-factor VIII, metabolically labelled des-(868–1562)-factor VIII-R2307Q was not encountered in the conditioned medium of the transfected cells, indicating that the mutant protein is not secreted from the cell. Inspection of the intracellular localization of the two proteins in the cell employing morphological analysis, endoglycosidase H and experiments with inhibitors of glucosidases I and II was consistent with localization of des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q in the endoplasmic reticulum. Taken together, our data indicate that the Arg²³⁰⁷ → Gln mutation results in aberrant intracellular trafficking of factor VIII, which may explain the low levels of factor VIII antigen in the plasma of haemophilia A patients that carry this particular point mutation.

INTRODUCTION

Factor VIII functions in the intrinsic pathway of blood coagulation as a cofactor, in the presence of Ca²⁺ and phospholipids, for the factor IXa-dependent conversion of factor X into factor Xa [1]. Molecular cloning of factor VIII cDNA revealed that factor VIII consists of a series of homologous domains which can be represented in the order A1–A2–B–A3–C1–C2 [2,3]. In plasma, factor VIII circulates as a metal-ion-linked heterodimer which is non-covalently bound to von Willebrand factor [4]. The heavy chain of factor VIII, with an *M_r* of 90 000–200 000, comprises the A1 and A2 domains as well as variable portions of the B domain [5]. The factor VIII light chain of *M_r* 80 000 consists of the A3, C1 and C2 domains [5]. Activation of factor VIII occurs by limited proteolysis by thrombin at amino acid positions Arg³⁷² and Arg⁷⁴⁰ in the heavy chain, and Arg¹⁶⁸⁹ in the light chain [6]. Consequently, activated factor VIII consists of a heterotrimer of the separate A1 and A2 domains together with a thrombin-cleaved light chain [7,8].

The X-linked bleeding disorder haemophilia A has been associated with the functional absence of factor VIII. Over the years, the molecular defect in the factor VIII gene of a large number of haemophilia A patients has been localized [9,10]. Particular interest has been directed at haemophilia A patients that contain cross-reacting material (CRM⁺) in their plasma [11]. Elegant studies employing micropurification of factor VIII from the plasma of CRM⁺-haemophilia A patients have contributed significantly to the current knowledge of the structure–function relationships of factor VIII [11–16]. Mutations at the thrombin cleavage sites at amino acids Arg³⁷² and Arg¹⁶⁸⁹ have been shown to interfere with the activation of factor VIII [12–16]. This is in agreement with data obtained with recombinant proteins in which the cleavage sites of thrombin were altered by site-directed mutagenesis [17]. Furthermore, two mutations in the factor VIII

gene were shown to result in additional glycosylation of the protein that interfered with factor VIII activity [18].

The molecular basis of the genetic defect in patients with a reduced level of factor VIII in their plasma has been explored to a lesser extent. Higuchi and co-workers have identified a mutation in the factor VIII gene of a patient suffering from mild haemophilia A that resulted in the substitution of a tyrosine for a phenylalanine at amino acid position 1680 [19]. A recombinant factor VIII protein containing this particular amino acid substitution was shown to be defective in binding to von Willebrand factor, which may explain the low levels of factor VIII in patients that carry the Tyr¹⁶⁸⁰ → Phe mutation [20]. Here, we have analysed a factor VIII molecule in which the arginine at amino acid position 2307 is replaced by a glutamine. This particular mutation has been associated with mild to moderate haemophilia A [21,22]. The functional importance of residue Arg²³⁰⁷ in factor VIII is suggested by the ability of a synthetic peptide containing this particular residue to inhibit binding of factor VIII to phospholipids [23,24]. Furthermore, evidence has been presented that this part of the factor VIII molecule is involved in binding to von Willebrand factor [25]. Initial attempts to characterize the molecular defect of the Arg²³⁰⁷ → Gln mutation using material purified from plasma have been unsuccessful [21]. We have introduced the Arg²³⁰⁷ to Gln substitution into the cDNA of a B-domain-deleted factor VIII and expressed the mutant cDNA in C127 cells.

MATERIALS AND METHODS

Materials

DNA-modifying enzymes, restriction enzymes, culture media, trypsin and antibiotics were obtained from Gibco (Breda, The

Abbreviations used: endo H, endoglycosidase H; ER, endoplasmic reticulum; IPB, immunoprecipitation buffer; PDI, protein disulphide isomerase.
* To whom correspondence should be addressed.

Netherlands). Oligonucleotide primers were synthesized using an automated DNA synthesizer (type 381A; Applied Biosystems, Foster City, CA, U.S.A.). The Sequenase kit was obtained from USB Corp. (Cleveland, OH, U.S.A.). *Pfu* polymerase was purchased from Stratagene (Cambridge, U.K.). Radioactive chemicals were obtained from Amersham International (Amersham, Bucks., U.K.). Monoclonal antibody ESH-5 directed against the heavy chain of factor VIII was from American Diagnostica (Greenwich, CT, U.S.A.). Protein A-Sepharose CL-4B, gelatin-Sepharose 4B and the plasmid pBPV were obtained from Pharmacia-LKB (Uppsala, Sweden). Endoglycosidase H (endo H) and castanospermine were purchased from Boehringer Mannheim (Mannheim, Germany). Immunoaffinity-purified antibody raised against bovine protein disulphide isomerase (PDI) was kindly provided by Professor R. B. Freedman (Department of Biochemistry, University of Kent, Canterbury, U.K.).

Plasmid constructions

The plasmid pCLB-BPVdB695, encoding des-(868–1562)-factor VIII, has been described previously [factor VIII-del(868–1562) in [26]]. Plasmid pCLB-BPVdB695 was modified by introducing a synthetic linker (5' TCGACCTCCAGTTGAACATTTGTAG-CAAGCCACCATGGAAATAGAGCT 3'), containing part of the 5' untranslated region of the factor VIII cDNA linked to a consensus sequence for initiation of translation, in front of the start codon (underlined) at the 5' end of the factor VIII cDNA [27]. At the 3' end a linker (5' CTGGAGGGTTCGACCTGCA-GGCATGCCTCGAGCCGCGGCCGC 3') was inserted in the *Pst*I site at nucleotide position 7066 of the factor VIII cDNA (nucleotide 1 corresponds to the first nucleotide of the start codon). The *Nor*I site was utilized to clone the des-(868–1562)-factor VIII cDNA into plasmid pBPV, yielding plasmid pCLB-dB695.

The plasmid pCLB-dB695-R2307Q was constructed by site-directed mutagenesis using overlap extension [28]. Oligonucleotide primer RQ2307-1 (5' CGCTACCTTCAATTCAC-CCC 3'; nucleotides 6967–6987 of factor VIII; sense) and oligonucleotide primer RQ2307-2 (5'CCATAGGTTGGAATC-TAA 3'; nucleotides 1221–1238 of pBPV; antisense) were used to amplify a 286 bp fragment that contained part of the factor VIII cDNA and the plasmid pBPV. Oligonucleotide primer RQ2307-3 (5' TTAGGATCCCACTAAAGATGAGTTT 3'; nucleotides 5532–5547; sense) was used together with oligonucleotide primer RQ2307-4 (5' GGGGTGAATTGGAAGGTAGCG 3'; nucleotides 6967–6987 of factor VIII; antisense) to amplify a 1455 bp fragment of factor VIII. Reaction conditions were: 2 min at 90 °C, 20 min at 50 °C, 3 min at 72 °C; followed by 37 cycles of 45 s at 90 °C, 90 s at 50 °C, 3 min at 72 °C; and finally 5 min at 65 °C in the presence of 1 mM dNTPs, *Pfu* polymerase reaction buffer, 50 pmol of each oligonucleotide primer and 2.5 units of *Pfu* polymerase [29]. Both the 286 bp and 1455 bp fragments were purified and used as templates in a PCR using primers RQ2307-1 and RQ2307-4 in order to amplify a 1723 bp fragment of cDNA encoding the C-terminus of factor VIII in which Arg²³⁰⁷ has been replaced by Gln. The amplified fragment was digested with *Sal*I and *Apa*I, and the resulting 872 bp fragment containing a mutation corresponding to amino acid position Arg²³⁰⁷ was used to replace the corresponding *Apa*I–*Sal*I fragment of pCLB-dB695. The resulting construct was termed pCLB-dB695-R2307Q. The nucleotide sequence of the *Sal*I–*Apa*I fragment used for the construction of pCLB-dB695-R2307Q was verified.

Tissue culture and transfection

C127 cells were maintained in Iscove's medium supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Subconfluent monolayers of C127 cells were transfected essentially as described previously [30]. The presence of factor VIII protein in the culture medium was monitored by measuring both factor VIII activity and factor VIII antigen, as described previously [26]. Factor VIII cofactor activity was assessed by the ability of factor VIII to function as a cofactor for the factor IXa-dependent formation of factor Xa, employing a chromogenic substrate for factor Xa (Coatest Factor VIII; Chromogenix, Mölndal, Sweden). Factor VIII antigen was determined using monoclonal antibodies that have been characterized previously [31,31a]. Monoclonal antibody CLB-CAG A, directed against the factor VIII light chain, was used as a solid phase; peroxidase-labelled monoclonal antibody CLB-CAG 58, also directed against the light chain, was used to quantify the amount of factor VIII bound. Normal plasma from a pool of 40 donors was used as a standard. The amount of factor VIII heavy chain was determined as follows. Monoclonal antibody ESH-5 (2.5 µg/ml) was immobilized to microtitre wells overnight at 4 °C in 50 mM NaHCO₃ (pH 9.5). Wells were washed with PBS/0.1% (v/v) Tween-20. Factor VIII samples were diluted into 50 mM Tris/HCl (pH 7.4), 2% (v/v) human serum albumin and 1 M NaCl, and incubated for 2 h at 37 °C with the immobilized antibody. Subsequently, the wells were washed and the amount of factor VIII heavy chain bound was determined by employing peroxidase-labelled monoclonal antibody CLB-CAG 9 at a concentration of 0.7 µg/well in 50 mM Tris/HCl (pH 7.4), 0.2% (v/v) Tween-20 and 0.15 M NaCl. The detection limit of this particular assay is 45 m-units/ml. Isolated factor VIII heavy chain was used as a standard. The immunofluorescence of transfected cells employing the monoclonal antibodies CLB-CAG A, CLB-CAG 58, CLB-CAG 9 and ESH-5 revealed that these antibodies react with both des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q.

Metabolic labelling and immunoprecipitation

Transfected cells, maintained in Iscove's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin, were metabolically labelled upon reaching 80% confluence. Cells were washed twice with PBS and incubated for 30 min in RPMI lacking methionine. Subsequently, the cells were labelled for 30 min in the same medium supplemented with 10% fetal calf serum (dialysed against 25 mM Hepes, pH 7.0) and [³⁵S]methionine (50 µCi/ml; sp. radioactivity > 800 Ci/mmol). Following metabolic labelling, cells were chased for various times in the presence of RPMI supplemented with 10% fetal calf serum. The medium of metabolically labelled cells was collected in an equal volume of 2× concentrated immunoprecipitation buffer (IPB) consisting of 1% Nonidet P-40, 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.5% SDS, 10 µg/ml soybean trypsin inhibitor, 10 mM benzamidine and 5 mM *N*-ethylmaleimide. Cells were washed twice with PBS and lysed in IPB. Lysates and conditioned media were either stored at –20 °C or used immediately for immunoprecipitation. Cell extracts and conditioned media were precleared by incubation for 1 h at room temperature with gelatin-Sepharose 4B and two successive incubations with Protein A-Sepharose CL-4B. Specific adsorption was performed overnight at 4 °C, using preformed complexes of Protein A-Sepharose CL-4B and polyclonal rabbit antiserum raised against factor VIII purified from plasma. Immunoprecipitates were extensively washed with IPB and finally with 20 mM Tris/HCl (pH 7.4), and analysed under reducing conditions on an

SDS/7.5% (w/v)-polyacrylamide gel. Following electrophoresis, gels were fixed in 30% methanol/10% acetic acid and treated with 20% diphenylloxazol in acetic acid for 30 min. Subsequently, gels were incubated in water, dried and exposed for variable times. Endo H digestions of immunoprecipitated material were performed as described previously [32].

Immunofluorescence studies

C127 cells transfected with des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q cDNA were grown on coverslips coated with 1% gelatin. At confluence cells were fixed for 10 min with methanol. Following fixation cells were washed twice with PBS and incubated with monoclonal or polyclonal antibodies. Monoclonal antibody CLB-Cag 12 and an immunoaffinity-purified antibody raised against homogeneous bovine PDI were diluted 1:100 in PBS/2% (v/v) BSA and incubated for 1 h. The coverslips were washed three times with PBS and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum and tetramethyl rhodamine isothiocyanate-conjugated goat anti-mouse antiserum, diluted 1:100 in PBS/2% (v/v) BSA. Coverslips were prepared for microscopy as described previously [33].

RESULTS

Expression of des-(868–1562)-factor VIII-R2307Q in C127 cells

Previously, we have described the expression of des-(868–1562)-factor VIII, a B-domain-deleted form of factor VIII, in C127 cells [26]. Extensive biochemical characterization of the expressed protein revealed that it behaves identically to factor VIII purified from plasma. Assessment of the properties of this molecule *in vivo* in an animal model of haemophilia A yielded haemostatic parameters similar to those observed for plasma-derived factor VIII. In order to define the molecular defect observed in haemophilia A patients that carry a substitution of Arg²³⁰⁷ by Gln, we have introduced this mutation into the cDNA of des-(868–1562)-factor VIII and expressed the mutant cDNA in C127 cells (Figure 1). Transfected cells were analysed for secretion of factor VIII, both by measurement of factor VIII cofactor activity and by determining factor VIII antigen. In contrast to des-(868–1562)-factor VIII, only a limited amount of cofactor activity could be detected in the medium of cells transfected with des-

Table 1 Analysis of conditioned medium of transfected cells for the presence of factor VIII cofactor activity and factor VIII antigen

Cofactor activity was determined as outlined in the Materials and methods section. The presence of factor VIII light chain and heavy chain was addressed using monoclonal antibodies as described in the Materials and methods section. A pool of normal plasma from 40 healthy individuals was used as a standard for cofactor activity and for determining the amount of factor VIII light chain in the conditioned medium. A preparation of purified factor VIII heavy chain was used as a standard for determining the amount of factor VIII heavy chain. Values are means $\pm$ S.D., and are expressed in m-units/ml.

Parameter	Des-(868–1562)-factor VIII	Des-(868–1562)-factor VIII-R2307Q
Cofactor activity	181 $\pm$ 2	1.73 $\pm$ 0.06
Antigen (light chain)	214 $\pm$ 16	< 10
Antigen (heavy chain)	187 $\pm$ 14	< 45

(868–1562)-factor VIII-R2307Q cDNA (Table 1). The amount of factor VIII light chain in the conditioned medium of cells transfected with des-(868–1562)-factor VIII-R2307Q was below the detection limit of the assay (Table 1). Factor VIII heavy chain in the conditioned medium was determined using monoclonal antibody CLB-Cag 9, directed against amino acid sequence Lys⁷¹³–Arg⁷⁴⁰ of factor VIII, in conjunction with monoclonal antibody ESH-5 [34,35]. No factor VIII antigen was present in the medium of cells that had been stably transfected with des-(868–1562)-factor VIII-R2307Q cDNA, while des-(868–1562)-factor VIII could be detected using these combinations of antibodies (Table 1). The results obtained clearly show that, compared with des-(868–1562)-factor VIII, only limited amounts of des-(868–1562)-factor VIII-R2307Q are present in the conditioned medium of cells transfected with des-(868–1562)-factor VIII-R2307Q cDNA.

Pulse-chase analysis of transfected cells

In order to monitor the biosynthesis of both des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q, stably transfected cells expressing these proteins were metabolically labelled. Factor VIII was immunoprecipitated from the metabolically labelled cells using a polyclonal anti-factor VIII antibody. Analysis of cell extracts of metabolically labelled cells transfected with des-(868–1562)-factor VIII cDNA revealed a prominent

Figure 1 Schematic representation of des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q

Cleavage sites for thrombin at amino acids Arg³⁷², Arg⁷⁴⁰ and Arg¹⁶⁸⁹ are indicated by arrows. Proteolytic cleavage at Arg¹⁶⁴⁸ by an as yet unidentified protease occurs during biosynthesis of des-(868–1562)-factor VIII and results in a light chain of M_r 80 000 (domains A3–C1–C2) and a heavy chain (domains A1–A2–B) which contains part of the B domain of factor VIII. In des-(868–1562)-factor VIII, amino acids Ala⁸⁶⁸–Leu¹⁵⁶² have been deleted. The region of the C2 domain around amino acid Arg²³⁰⁷ is depicted in the box for both des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q.

Figure 2 Pulse-chase analysis of cells transfected with des-(868–1562)-factor VIII cDNA

Cells were metabolically labelled for 30 min with [35 S]methionine and chased for 0, 1, 2, 4 and 6 h. Cell extracts and conditioned media were harvested, immunoprecipitated with a polyclonal antibody directed against factor VIII and analysed on an SDS/7.5% (w/v)-polyacrylamide gel. As a control, non-transfected cells were employed. **(A)** Analysis of cell extracts of transfected cells at different periods of chase: lane 1, 0 h; lane 2, 1 h; lane 3, 2 h; lane 4, 4 h; lane 5, 6 h; lane 6, control. **(B)** Analysis of conditioned medium at different periods of chase (lanes as in **A**). The single chain (sc), light chain (lc) and heavy chain (hc) of factor VIII are indicated in the figure. M_r markers ($10^{-3} \times M_r$) are indicated on the right.

band with an M_r of 180 000 that gradually disappeared from the cells with time (Figure 2A). Two less intense bands that migrated somewhat faster were present only at the beginning of the chase. Concomitant with the decrease in intensity of the band of approx. M_r 180 000, which most likely represents the single-chain form of des-(868–1562)-factor VIII, an increase in immunoreactive material was observed in the medium (Figure 2B). In agreement with previous data [26], des-(868–1562)-factor VIII was secreted in part as a single-chain protein migrating at an M_r of 180 000. Two additional bands migrating at M_r s of 80 000 and 130 000 were visible in the conditioned medium (Figure 2B). The band with an M_r of 80 000 corresponds to the factor VIII light chain, while the band of approx. M_r 130 000 corresponds to the heavy chain, which contains part of the B-domain of des-(868–1562)-factor VIII [26]. Our results are consistent with proteolytic processing of a significant part of des-(868–1562)-factor VIII at amino acid Arg¹⁶⁴⁸ (Figure 1).

Cell extracts of metabolically labelled cells that had been stably transfected with des-(868–1562)-factor VIII-R2307Q were analysed as described above. Des-(868–1562)-factor VIII-R2307Q was synthesized as a single-chain molecule with an M_r of 180 000 (Figure 3A). In accordance with data obtained for des-(868–1562)-factor VIII, two less intense bands migrating somewhat faster than the single-chain factor VIII were present at the beginning of the chase. The amount of des-(868–1562)-factor VIII-R2307Q synthesized during the pulse was similar to that of des-(868–1562)-factor VIII, as determined by densitometric scanning of autoradiographs [ratio of synthesized des-(868–1562)-factor VIII to des-(868–1562)-factor VIII-R2307Q was 1.43 ± 0.52 ; mean $\pm$ S.D.; $n = 7$). This observation indicates that the two proteins are expressed to a similar extent in transfected C127 cells. Compared with des-(868–1562)-factor VIII, the single-chain form of des-(868–1562)-factor VIII-R2307Q seemed to persist somewhat longer in the transfected cells. Immunoprecipitation of conditioned medium of metabolically labelled cells that had been stably transfected with des-(868–1562)-factor VIII-R2307Q failed to recover any immunoreactive material (Figure 3B). This observation suggests that des-(868–1562)-factor

Figure 3 Pulse-chase analysis of cells transfected with des-(868–1562)-factor VIII-R2307Q cDNA

Transfected cells were metabolically labelled for 30 min using [35 S]methionine. The cells were then incubated in complete medium for different periods of time and analysed as described for Figure 2. **(A)** Analysis of cell extracts at different times of chase: lane 1, 0 h; lane 2, 1 h; lane 3, 2 h; lane 4, 4 h; lane 5, 6 h; lane 6, control. **(B)** Analysis of conditioned medium at different periods of chase: lane 1, 0 h; lane 2, 2 h; lane 3, 4 h; lane 4, 6 h; lane 5, 8 h; lane 6, control. M_r markers ($10^{-3} \times M_r$) are indicated on the right, and single-chain des-(868–1562)-factor VIII-R2307Q (sc) is indicated on the left.

VIII-R2307Q is not secreted from the cell and indicates that the vast majority of the initially synthesized des-(868–1562)-factor VIII-R2307Q is processed within the cell via a mechanism different from that for des-(868–1562)-factor VIII.

Biochemical analysis of intracellular factor VIII

In the previous section we showed that des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q are synthesized to the same extent in transfected C127 cells. In contrast to des-(868–1562)-factor VIII, des-(868–1562)-factor VIII-R2307Q is not found in the conditioned medium of transfected cells, suggesting a biosynthetic defect of the latter protein. In order to assess the intracellular localization of both des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q, immunoprecipitated proteins were tested for sensitivity to endo H. Transfected cells were labelled for 30 min with [35 S]methionine and chased for different periods of time. Factor VIII was recovered from the cell extracts by immunoprecipitation and incubated with endo H. Following 30 min of labelling, immunoprecipitated des-(868–1562)-factor VIII was sensitive to the addition of endo H; a small shift in M_r was observed upon incubation of the single-chain des-(868–1562)-factor VIII with endo H (Figure 4A, lanes 1 and 2). Inspection of endo H sensitivity at different time points revealed that des-(868–1562)-factor VIII remained sensitive to endo H throughout the experiment. Analysis of des-(868–1562)-factor VIII-R2307Q revealed that this protein, like des-(868–1562)-factor VIII, was sensitive to endo H at the different time points indicated. These results indicate that both des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q are predominantly present in a compartment prior to the medial Golgi.

To further assess the intracellular localization of des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q in the transfected cells, the sensitivity of the single-chain proteins to inhibitors of the endoplasmic reticulum (ER)-localized enzymes glucosidases I and II was tested. Cells were labelled for 1 h in the presence of 1 mM castanospermine (an inhibitor of glucosidase I) or 10 μ M *N*-methyl-1-deoxynojirimycin (an in-

Figure 4 Endo H digestion of des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q

Transfected cells were metabolically labelled with [35 S]methionine for 30 min, followed by incubation in complete medium for various times. Cell extracts were harvested, immunoprecipitated and digested with endo H as described [32]. Samples digested with endo H and undigested samples were analysed by SDS/7.5%-PAGE. **(A)** Cells transfected with des-(868–1562)-factor VIII cDNA: lane 1, 0 h chase + endo H; lane 2, 0 h chase (no endo H); lane 3, 2 h chase + endo H; lane 4, 4 h chase + endo H; lane 5, 6 h chase + endo H. **(B)** Cells transfected with des-(868–1562)-factor VIII-R2307Q cDNA: lane 1, 0 h chase (no endo H); lane 2, 0 h chase + endo H; lane 3, 2 h chase + endo H; lane 4, 4 h chase + endo H; lane 5, 6 h chase + endo H. The arrows indicate the endo H-modified proteins. M_r markers ($10^{-3} \times M_r$) are indicated on the right.

Figure 5 Heterogeneity of des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q

Transfected cells were metabolically labelled for 1 h with [35 S]methionine in the presence of 1 mM castanospermine or 10 μ M *N*-methyl-1-deoxyojirimycin. Following metabolic labelling, cell extracts were harvested and immunoprecipitated with a polyclonal antibody directed against factor VIII, and analysed by SDS/7.5%-PAGE. **(A)** Analysis of cells transfected with des-(868–1562)-factor VIII: lane 1, incubation with 1 mM castanospermine; lane 2, incubation with 10 μ M methyldeoxyojirimycin; lane 3, control. **(B)** Analysis of cells transfected with des-(868–1562)-factor VIII-R2307Q (lanes as for **A**). An M_r marker ($10^{-3} \times M_r$) is indicated on the right. Only the relevant portion of the gel is shown.

inhibitor of both glucosidases I and II [35]). Addition of castanospermine (Figure 5A, lane 1) or *N*-methyl-1-deoxyojirimycin (Figure 5A, lane 2) during metabolic labelling of cells transfected with des-(868–1562)-factor VIII cDNA resulted in an increase in mobility of the single-chain factor VIII compared with in the non-treated cells (Figure 5, lane 3). From the observed shift in M_r it can be concluded that, in the absence of inhibitors of glucosidases I and II, the single-chain des-(868–1562)-factor VIII is, at least partially, processed by these enzymes. It should be noted that the mobilities of the two bands that migrated slightly faster than the single-chain des-(868–1562)-factor VIII were insensitive to the addition of castanospermine and *N*-methyl-1-

deoxynojirimycin. Presumably these forms of factor VIII correspond to the addition of inhibitors of glucosidases I and II. Inspection of the sensitivity of single-chain des-(868–1562)-factor VIII-R2307Q to castanospermine and *N*-methyl-1-deoxyojirimycin revealed a pattern similar to that observed for des-(868–1562)-factor VIII (Figure 5B). These results indicate that both des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q are processed, at least in part, by glucosidases I and II.

Immunolocalization of factor VIII in transfected cells

We have shown that the majority of the intracellular des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q is sensitive to endo H and is processed by ER-localized glucosidases. To confirm the intracellular localization of the two proteins, transfected cells were stained by immunofluorescence employing monoclonal antibody CLB-CAG 12 directed against the light chain of factor VIII [31]. Inspection of the pattern obtained for cells transfected with des-(868–1562)-factor VIII cDNA revealed staining of a reticular network (Figure 6A). Double labelling with a polyclonal antibody directed against the resident ER protein PDI was also performed [36]. The pattern observed was similar to that observed for des-(868–1562)-factor VIII, indicating that the latter was localized in the ER of the transfected cells (Figure 6B). Subsequently, C127 cells that had been stably transfected with des-(868–1562)-factor VIII-R2307Q cDNA were analysed by immunofluorescence. Monoclonal antibody CLB-CAG 12 yielded a pattern similar to that observed for cells stably transfected with des-(868–1562)-factor VIII cDNA (Figure 6C). Double staining employing a polyclonal antibody against PDI revealed that des-(868–1562)-factor VIII-R2307Q was also present in the ER (Figure 6D). In accordance with the biochemical data obtained for digestion with endo H, these results provide evidence for the localization of both des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q in the ER of the transfected cells.

DISCUSSION

The results described here indicate that substitution of the arginine at amino acid position 2307 by glutamine results in the aberrant biosynthesis of factor VIII. This phenomenon may explain the mild to moderate bleeding diathesis observed in haemophilia A patients that carry this Arg²³⁰⁷ → Gln mutation [10,21,22]. Careful analysis employing monoclonal antibodies directed against both the heavy and light chains of factor VIII did not reveal immunoreactive material in the medium of cells that had been stably transfected with des-(868–1562)-factor VIII cDNA containing the Arg²³⁰⁷ to Gln mutation (Table 1). Identical conclusions were drawn from immunoprecipitation experiments (Figure 3). Our data indicate that, under the conditions employed, no immunoreactive material is secreted by stably transfected cells. It should be noted, however, that a small but significant amount of factor VIII cofactor activity was found in the conditioned medium of cells transfected with des-(868–1562)-factor VIII-R2307Q cDNA (Table 1). Since the amount of factor VIII antigen in the conditioned medium could not be determined, we do not know whether the small amount of des-(868–1562)-factor VIII-R2307Q secreted is fully active. Micropurification of immunoreactive material derived from the plasma of a patient carrying the Arg²³⁰⁷ → Gln mutation has been reported, and the resulting purified preparation was found to contain procoagulant activity and could be activated by thrombin [21]. Together with the results presented in the present paper, this may suggest that

Figure 6 Morphological analysis of cells transfected with des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q cDNA

Transfected cells were grown to confluence on coverslips, fixed with methanol and incubated with monoclonal antibodies directed against factor VIII and with a polyclonal antibody directed against PDI. (A) Cells transfected with des-(868–1562)-factor VIII cDNA and stained with CLB-CAG 12. (B) Same cell as depicted in (A) now stained with anti-PDI antibodies. (C) Cells transfected with des-(868–1562)-factor VIII-R2307Q cDNA and stained with CLB-CAG 12. (D) Same cell as in (C) but now stained with anti-PDI antibodies. Magnification 1250 $\times$.

the Arg²³⁰⁷ $\rightarrow$ Gln mutation does not result in a dysfunctional protein, but merely causes intracellular retention of factor VIII.

The aberrant biosynthesis of des-(868–1562)-factor VIII-R2307Q may indicate that this particular protein is assembled incorrectly. Both immunofluorescent staining and endo H digestions are consistent with the localization of des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q in the ER of the cell. This is in accordance with data on the intracellular localization of factor VIII in CHO cells transfected with factor VIII cDNA [37,38]. It is of interest that immunoprecipitation of both des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q revealed three prominent bands at chase time zero. The origin of the two low- M_r bands is difficult to explain, but it is noteworthy that these bands are most prominent at the onset of chase, which suggests that they represent a form of factor VIII with a relatively short half-life. Both low- M_r forms of factor VIII are insensitive to the addition of castanospermine or *N*-methyl-1-deoxyjirimycin, suggesting that the terminal glucose residues have not been removed from these forms of factor VIII. In a recent study a similar heterogeneity was observed upon expression of the A1 domain of factor VIII in COS-1 cells [39]. The presence of terminal glucose residues has been associated with degradation of misfolded proteins that originate in the ER [40]. Whether these mechanisms also operate on factor VIII is an issue that is currently under investigation. It should be noted that des-(868–1562)-factor VIII and des-(868–1562)-factor VIII-R2307Q display an identical heterogeneity, which suggests that the intracellular retention and degradation of des-(868–1562)-factor VIII-R2307Q is not related to this phenomenon.

The experiments performed clearly show that, compared with des-(868–1562)-factor VIII, des-(868–1562)-factor VIII-R2307Q is poorly secreted by the transfected cells. This observation suggests that the presence of Arg²³⁰⁷ is required for efficient secretion of factor VIII. Inspection of murine factor VIII cDNA

and of human and bovine factor V cDNAs shows that Arg²³⁰⁷ is a highly conserved residue [41,42]. The functional importance of this residue in binding to phospholipids is suggested by the fact that peptides that correspond to the region Thr²³⁰³–Glu²³²⁴ inhibit the binding of factor VIII to phospholipids [23]. Recently, the three-dimensional structure of a peptide encompassing Thr²³⁰³–Leu²³²⁶ has been determined in the presence of SDS micelles [24]. From the data a model has been derived for the binding of this peptide to phospholipid membranes, and Arg²³⁰⁷ has been proposed to interact with the phosphate groups that are part of the phospholipid bilayer. Whether the intracellular retention of des-(868–1562)-factor VIII-R2307Q is related to the phospholipid-binding properties of this region of factor VIII remains to be established.

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