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Peptide affinity chromatography of human clotting factor VIII Screening of the vWF-binding domain

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Abstract

The region of von Willebrand factor, which is involved in the complex formation with factor VIII, was used to generate a panel of octapeptides. A peptide ladder was generated from the von Willebrand factor region aa40 to aa100 and was synthesized on cellulose membranes by spot technology. Four peptides with affinity for factor VIII were identified by incubation with plasma derived factor VIII and recombinant factor VIII. The peptides denoted as 010 (LCPPGMVRHE), 011 (RCPCFHQGK), 014 (CFHQGKEYA) and 015 (RDRKWNCTDHVC) were further characterized by real-time interaction analysis and small scale affinity chromatography. Biotinylated peptides were used for blotting assays. These experiments showed that the peptides are directed against the light chain of FVIII. We consider these peptides as valuable tools for in situ labeling and also as ligands suitable for affinity chromatography.

Keywords: Peptides; Factor VIII; Von Willebrand factor

1. Introduction

Affinity techniques are necessary for rapid isolation and detection of clotting factor VIII (FVIII). Natural complexes of FVIII and accompanying proteins, such as von Willebrand factor (vWF), antibodies or factor IXa (FIXa), can serve as sources for deriving small peptides with affinity for FVIII.

During circulation in plasma, FVIII is complexed with vWF, a high multimeric protein, by non-covalent interactions [1]. Both proteins are highly glycosilated and play a pivotal role in hemostasis [2]. FVIII is synthesized as a 300 000 single-chain molecule, consisting of six regions called A1–A2–B–A3–C1–C2, which show some homology [3]. FVIII was isolated in a two-chain form, composed of a light chain (A3–C1–C2) and a heavy chain (A1–A2–B) linked together by a metal bridge [4]. vWF is a 270 000 glycoprotein that assembles to multimers up to a molecular size equivalent to several millions of relative mass units. These multimers are formed through disulfide bridges and non-covalent interactions [5]. The vWF multimers interact very strongly with FVIII and form a non-covalent complex. Only under harsh conditions, such as the presence of reducing or chaotropic agents and high ionic strength, can the vWF–FVIII complex be dissociated [4]. Free FVIII is very unstable [6] and shows reduced half-life in plasma, as observed in patients suffering from some forms of von Willebrand disease where vWF–FVIII interaction is disturbed or reduced [7,8].

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Several groups described the FVIII region interacting with vWF by using inhibition assays with antibodies and peptides. The vWF-binding region on FVIII was localized between amino acid (aa)1670 and aa1689 [9–11]. New studies determined a vWF-binding region in the C2 domain of FVIII [12–14]. According to Leyte et al. [15] and Pittman and co-workers [16,17] Tyr in position 1680 must be sulfated in order to obtain full FVIII activity during blood coagulation. The binding affinity of FVIII for vWF will be significantly reduced, if the Tyr at aa1680 is not sulfated. The vWF region which binds to FVIII was identified within the region spanning the 272 N-terminal amino acids [18–20]. Further inhibition assays with peptides representing fragments of vWF reduced the epitopes to the region between aa10 and aa80 [21].

The chemical nature of the vWF–FVIII complex can be delineated by means of peptide mapping. Strategies and theoretical approaches for design, synthesis and deconvolution of peptide libraries opened a new dimension in the work with peptides. Ongoing developments in the area of peptide synthesis, miniaturization and the advancing degree of automation allow the synthesis of a large number of peptides on a relatively small scale [22–24]. Further improvements in parallel multiple peptide synthesis have made a large panel of peptides available for biological applications without time-intensive and cost-intensive work. During the last 15 years, different methods for building large peptide libraries, such as pin synthesis [25], teabag synthesis [26] and spot synthesis [27], have been developed. Parallel to this, methods for analyzing thousands or even millions of different compounds have been established [28]. Common to all library constructs designed as solid-phase devices is the immobilization of the peptides via a linker to the matrix, which serves as a support for the synthesis. For further investigations, these peptides can either be cleaved from the support or the assay is carried out directly on the solid-phase.

In this study, the potential region of vWF considered to interact with FVIII was investigated by peptide mapping. A peptide ladder with octamers ranging from aa40–aa100 was created by the spot technology described by Frank [27]. Several peptides with affinity for FVIII were identified and four candidates were further characterized with real-time measurements of the interactions utilizing surface plasmon resonance (SPR), affinity chromatography by immobilizing the candidates to pre-activated chromatography media, and various blotting techniques.

2. Experimental

2.1. Plasma-derived FVIII concentrates

Peptides synthesized onto cellulose membranes were incubated with plasma derived (pd)FVIII (2 μg/ml) in 0.1 *M* citrate buffer, pH 7.4 with 0.2 *M* NaCl and 1% bovine serum albumin (BSA). The pdFVIII was separated from the vWF–FVIII complex as described by Josic et al. [29] using the pdFVIII concentrate Octavi (Octapharma Pharmazeutika, Vienna, Austria).

All chromatographic experiments were performed using the pdFVIII formulation Octavi. After reconstitution of the lyophilisated pdFVIII concentrate, dilution with 10 m*M* 4-(2-hydroxyethyl)-1-piperazinethansulfonic acid (HEPES) pH 7.4 containing 5 m*M* $CaCl_2$ and 0.1% Triton X-100 to a final concentration of 140 international units (I.U.s) of FVIII/ml was carried out. The vWF–FVIII complex was dissociated by incubation with 40 m*M* dithiothreitol (DTT) for 1 h at 4°C.

For real-time measurements of the interactions utilizing SPR the pdFVIII concentrate Octavi SDP (Octapharma Pharmazeutika) was first dissolved in 10 m*M* HEPES, pH 7.4 containing 5 m*M* $CaCl_2$ and 0.1% Triton X-100, and then treated with DTT.

2.2. Recombinant FVIII

The screening of the spot membranes was performed with recombinant (r)FVIII (2 μg/ml) from Bayer (Berkely, CA, USA) in phosphate-buffered saline (PBS) containing 1% (w/v) BSA.

The loading solution for chromatographic experiments consisted of rFVIII (333 I.U. of FVIII/ml) in 10 m*M* HEPES, pH 7.4 containing 5 m*M* $CaCl_2$ and 0.1% Triton X-100.

2.3. Spot synthesis

The principle of spot synthesis as described by Frank [27] was used to map the region of FVIII which binds to vWF. The vWF region aa40 to aa100 was split into peptides eight amino acids long, with a shift of one amino acid from the N-terminus to the C-terminus (see Fig. 1). The amino acids were N-terminal 9-fluorenylmethoxycarbonyl (Fmoc)-protected and were purchased from Bachem (Bubendorf, Switzerland) and Novabiochem (Laeufelfingen, Switzerland). For the synthesis, the Auto-Spot Robot ASP 222 from Abimed (Langenfeld, Germany) was used. The carrier for the synthesis and the following assays was the Whatman 540 cellulose (Maidstone, UK) membrane. Hydroxyl groups of the cellulose membrane were derivatised by incubation with Fmoc-βAla and carbodiimide for 3 h. After the cleavage of the Fmoc group, the spots were defined by dispensing a defined volume (0.2 μl) of C-terminal-activated Fmoc-βAla onto the cellulose membrane. Each spot, an exactly defined area on the membrane, is used for the synthesis of the peptides with their individual sequence. Peptides were built up from the C-terminus to the N-terminus. The peptides were N-terminal acetylated after synthesis and the protecting side groups cleaved off. All membranes were equilibrated in PBS with 3% BSA overnight before starting the assays.

2.4. Screening

For screening the FVIII binding properties of the synthesized peptides, the cellulose membranes were incubated with both pdFVIII and rFVIII at a concentration of 2 μg/ml. After proper washing with PBS containing 1% BSA, a mixture of anti-FVIII antibodies containing monoclonal antibody (mAb) 038 (Chemicon, Temecula, CA, USA), mAb 530, mAb 531, and mAb 532 (Harlan-SeraLab, Sussex, UK) was added followed by the anti-mouse IgG alkaline phosphatase conjugate (Sigma, Deisenhofen, Germany). Peptides with affinity for FVIII were identified by adding the substrate nitroblue-tetra-

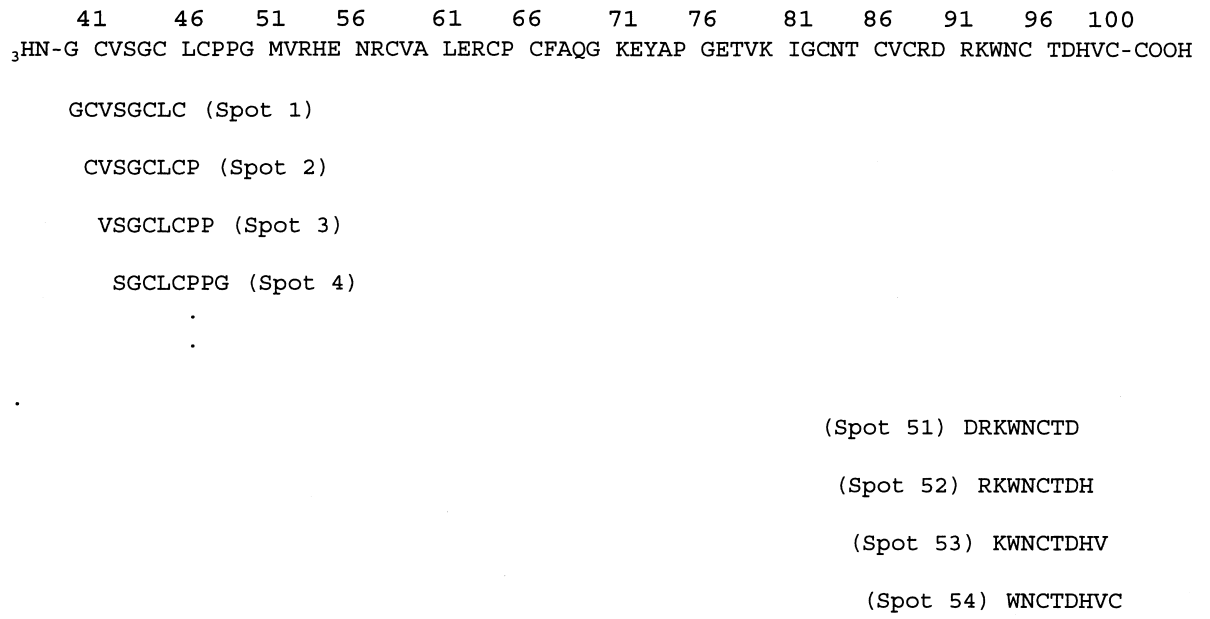

Fig. 1. Mapping of FVIII-binding region of vWF by synthesis of octapeptides with a shift of one amino acid from the C-terminus to the N-terminus.

zoliumchloride–5-bromo-4-chloro-3-indolylphosphate (NBT–BCIP) (Sigma).

2.5. Peptide synthesis

All peptides were synthesized with solid-phase peptide synthesis (SPPS) on a peptide synthesizer 431 A from ABI (Applied Biosystems, Foster City, CA, USA). The Fmoc amino acids were purchased from Bachem and the 4-hydroxymethylphenoxymethyl (HMP) resin from ABI. During the synthesis, the reactive side groups of the amino acids Cys, His, Asn and Gln were protected with trityl (trt), of Asp, Glu, Ser and Thr with *tert*.-butyl (tBu), of Lys with *tert*.-butoxycarbonyl (boc) and of Arg with pentamethylchroman sulfonyl (pmc).

The purity of the peptides was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) (HP 1090 liquid chromatography, Hewlett-Packard, Vienna, Austria) using a C_{18} column and a gradient from water to acetonitrile. The peptides were identified by their mass [matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), ThermoBioanalysis, Santa Fe, NM, USA].

2.6. Measurement of the interactions in real-time mode

Real-time biomolecular interaction analysis (BIA) of the immobilized candidate peptides and FVIII in solution were performed using a Biacore 2000 instrument (Biacore, Uppsala, Sweden). Utilizing the principle of SPR allows the detection of mass changes close to the dextran-coated surface (CM5) of the sensor chip without labeling one of the reaction partners. The candidate peptides were N-terminal immobilized onto the dextran layer after activation of its carboxyl groups with *N*-ethyl-*N′*-(dimethylaminopropyl)carbodiimide (EDC) and *N*-hydrosuccininide (NHS). The pdFVIII and rFVIII at a concentration of 350 or 333 I.U./ml, respectively, were pumped over the surface with the immobilized peptides. The binding of FVIII causes an increase in mass followed by changes of the SPR and an increase in the measured response units (RUs). The obtained data can be interpreted qualitatively as well as quantitatively.

2.7. Immobilization of peptides to pre-activated epoxy Sepharose FF

The candidate peptides were immobilized to pre-activated epoxy Sepharose FF (Pharmacia, Uppsala, Sweden) following the instructions provided by the supplier.

2.8. Chromatography

For all chromatographic experiments, the ProSys workstation (BioSepra, Marlborough, MA, USA) was used. A pump module containing four pumps and a separation module (sample applicator, chromatographic column and detectors for monitoring the values of UV, conductivity and pH) are connected to a computer which controls the workstation by a software supported by MS-Windows 3.1.

The UV signal was recorded at 280 nm. The HR 10 and HR 5 columns were from Pharmacia.

All buffers were degassed and filtered through a 0.22-μm filter (Millipore, Bedford, MA, USA).

2.9. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

For the separation of proteins according to Laemmli [30], an Xcell Mini-Cell system (Novex, San Diego, CA, USA) was filled with the standard 4–20% Tris–Glycine Novex pre-cast gels and operated according to the manual. The separated proteins were silver-stained.

2.10. Electroblotting

Proteins were transferred onto a nitrocellulose membrane (Protran Nitrocellulose Schleicher and Schuell, Dassel, Germany) using the Xcell Mini-Cell system. The BSA-blocked membranes were treated the same way the assaying of the spot membranes was performed (see Section 2.3).

2.11. Determination of the FVIII-binding region of the candidates

The candidate peptides were N-terminal conjugated with NHS pre-activated Biotin (Amersham, Little Chalfont, Buckinghamshire, UK) according to the manual. After incubation of the blot membrane carrying the electrophoretically-separated and electroblotted rFVIII with the conjugated peptides, the streptavidin–alkaline phosphatase-conjugate (Boehringer-Mannheim, Mannheim, Germany) was added. Biotinylated peptides were detected by adding the NBT–BCIP substrate.

2.12. Affinity chromatography

All four candidates were immobilized onto a preactivated epoxy Sepharose FF. Each affinity chromatography gel was packed into a glass column and connected to the chromatography workstation. The binding of vWF-free pdFVIII and rFVIII was investigated under chromatographic conditions. vWF-free pdFVIII was dissolved in 10 m*M* HEPES, pH 7.4 with 5 m*M* $CaCl_2$ and 0.1% (v/v) Triton X-100 and loaded onto the affinity column at a flow-rate of 60 cm/h. The bound material was eluted with a 0.1 *M* glycine buffer, pH 2.5 containing 1 *M* NaCl.

3. Results

The spot technology as originally described by Frank [27] was used to identify peptides with affinity for FVIII. A peptide ladder spanning the FVIII-binding region of vWF was synthesized on cellulose membranes. This approach allows us to use the same format for synthesis and further assays. Cellulose is known to have very low non-specific adsorption and its behavior in various blotting techniques is well characterized.

A peptide ladder consisting of 54 peptides was synthesized by splitting the FVIII-binding region of vWF (aa40 to aa100) into octapeptides with a shift of one amino acid. The amino acid sequences of the peptides and the peptide ladder are shown in Fig. 1. These peptides were synthesized in parallel on three different cellulose membranes denoted as A, B and C using the spot technology as described in Section 2.3.

For assaying the FVIII-binding properties of the peptides, the cellulose membranes were equilibrated in PBS containing 3% BSA. Membrane A was incubated with rFVIII and membrane B with vWF-free pdFVIII. After several washing steps, the membranes were incubated with the monoclonal mouse anti-FVIII antibodies as described in Section 2.4. After an additional washing step with alkaline phosphatase-conjugated anti-mouse IgG, the color reaction was developed by the substrate NBT–BCIP. As negative control, membrane C was treated identically, except for incubation with FVIII. In the comparison of spot intensity and characteristic color development, 11 spots with affinity for pdFVIII and more than 40 spots which bound rFVIII (see Fig. 2) were identified.

Four candidates with longer sequences such as those used in spot technology were derived from overlapping peptides after analysis of the spot membranes (see Table 1). These candidates were chemically synthesised and further characterized by real-time interaction analysis using SPR. For this purpose, the candidates were immobilized via their N-terminus onto the dextran-coated sensor chip using the NHS/EDC-mediated chemistry. rFVIII and pdFVIII were pumped over the surface with the immobilized peptides. The difference in absolute response before and after the injection was measured (see Fig. 3). All four candidates interacted with rFVIII and vWF-free pdFVIII. The candidates denoted as 010 and 014 showed the highest absolute response with both FVIII preparations.

All four candidates were immobilized as described in Section 2.6. Then their performance was checked by application of rFVIII and pdFVIII. The chromatograms are shown in Fig. 4. The eluates were collected and analyzed by SDS–PAGE and Western Blots. The fractions from the affinity chromatography gel with the immobilized candidates denoted as peptides 014 and 015 contained significant amounts of pdFVIII (see Fig. 5). As already indicated by the chromatograms, FVIII was not detected in the eluates of the immobilized peptides 010 and 011.

rFVIII, too, was dissolved in 10 m*M* HEPES, pH

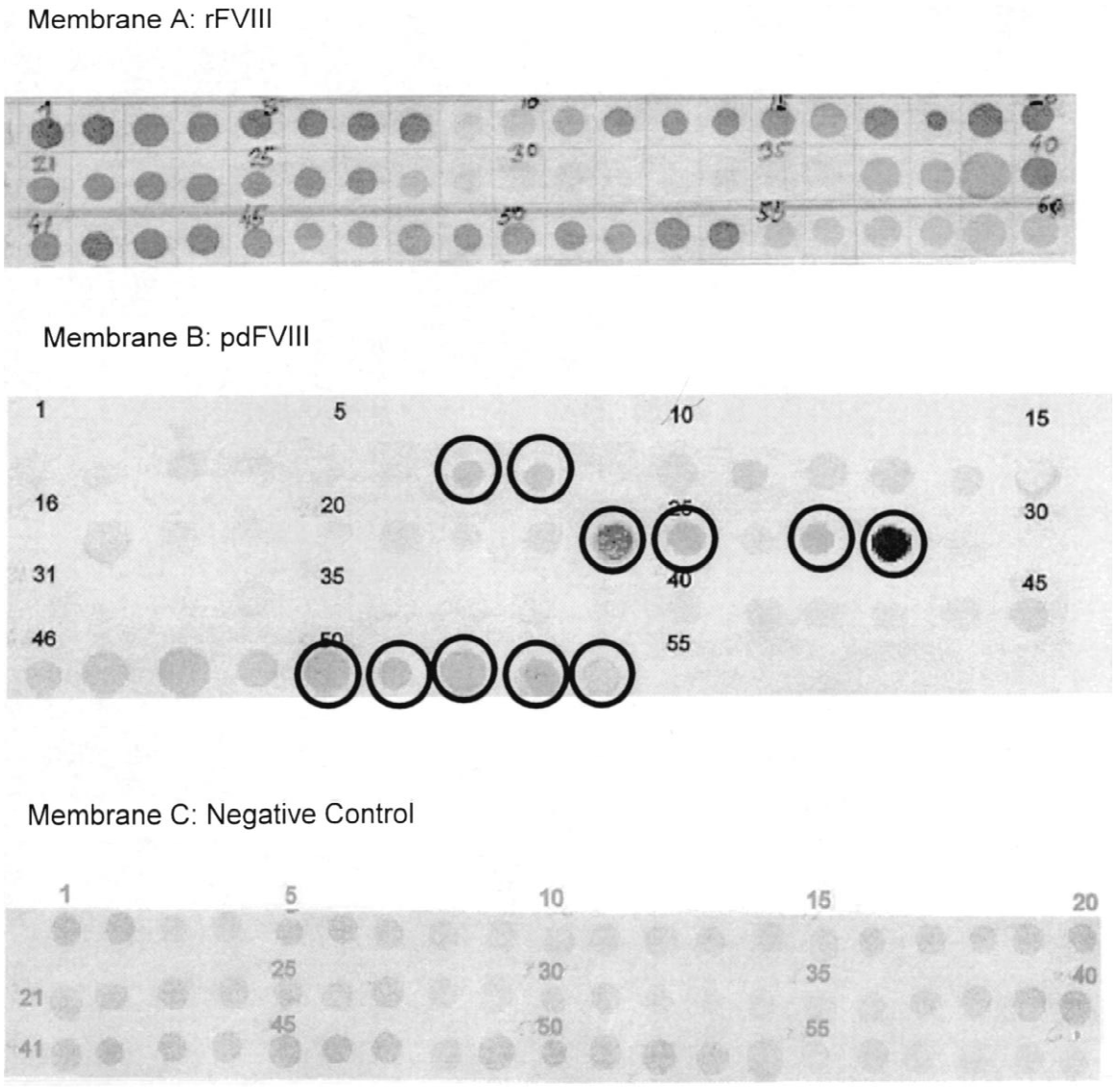

Fig. 2. Screening of FVIII-binding region of vWF for peptides with affinity for FVIII. Membrane A represents the cellulose sheet incubated with rFVIII, and membrane B that with pdFVIII followed by incubation with ant-FVIII mAbs and alkaline phosphatase-conjugated anti-mouse IgG. Positive spots, which represent peptides with affinity for FVIII, are circled. Membrane C (negative control) was treated identically but was not incubated with FVIII.

7.4 with 5 m*M* $CaCl_2$ and 0.1% Triton X-100 and loaded onto the column. The elution was carried out with a 0.1 *M* glycine buffer, pH 2.5 containing 1 *M* NaCl. The chromatograms are shown in Fig. 6. The eluates and the sample were analyzed by SDS–PAGE and Western Blot. Significant amounts of FVIII were detected by an immunoblot of the eluates from affinity chromatography gels with the immobilized candidates denoted as peptides 011, 014 and 015 (see Fig. 7).

rFVIII was electrophoretically separated and electroblotted onto a nitrocellulose membrane. The

Table 1
Physicochemical parameters of peptides which have affinity for FVIII and which were derived from mapping of the FVIII-binding region of vWF by spot synthesis

Internal code	Sequence	Sequence region of vWF	p*I*	Molecular mass	Spot number
010	LCPPGMVRHE	46–55	7.2	1138	7–8
011	RCPCFHQGK	63–71	8.9	1075	24–25
014	CFHQGKEYA	66–74	7.2	1087	27–28
015	RDRKWNCTDHVC	89–100	8.22	1533	50–54

candidate peptides were N-terminal biotinylated and used for incubation of the electroblotted rFVIII. The bound biotinylated peptides were detected by streptavidin–alkaline phosphatase conjugate and the NBT–BCIP substrate. The developed blots are shown in Fig. 8. Several bands were stained. The FVIII regions recognized by the peptides were localized in parallel experiments using monoclonal antibodies directed towards well-characterized epitopes of FVIII. The antibody 530p recognizes an epitope located on the heavy chain, whereas the mAb 532p reacts with the B-domain on the heavy chain of FVIII. Antibody 038 recognizes an epitope on the light chain [31]. The band pattern obtained by the biotinylated peptides is similar to the pattern of antibody 038 regarding the bands observed in the molecular mass range of 30 000–80 000. In addition, some stained bands in the high- and low-molecular-mass area were detected. A ≈200 000 band can be seen. This band has been also observed with antibody 530p.

The four candidates showed affinity for FVIII in

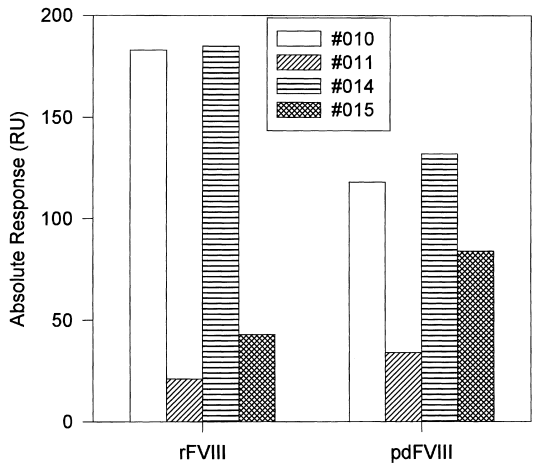

Fig. 3. Analysis of interaction between selected candidates and vWF-free pdFVIII or rFVIII, respectively, by real time interaction assay using the Biacore system.

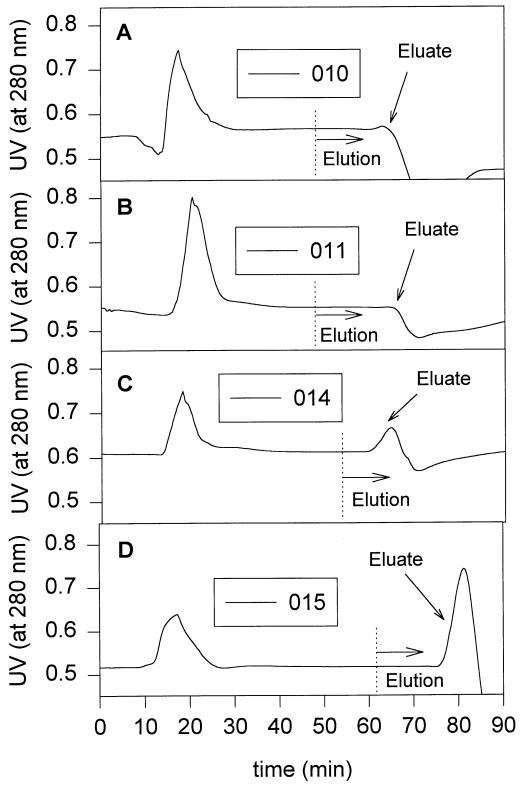

Fig. 4. Affinity chromatography with candidates immobilized onto epoxy Sepharose FF loading vWF-free pdFVIII. The pdFVIII concentrate Octavi was treated with DTT and loaded in 10 m*M* HEPES, pH 7.4 with 5 m*M* $CaCl_2$ and 0.1% (v/v) Triton X-100. (A) Peptide 010 (LCPPGMVRHE), (B) peptide 011 (RCPCFHQGK), (C) peptide 014 (CFHQGKEYA) and (D) peptide 015 (RDRKWNCTDHVC).

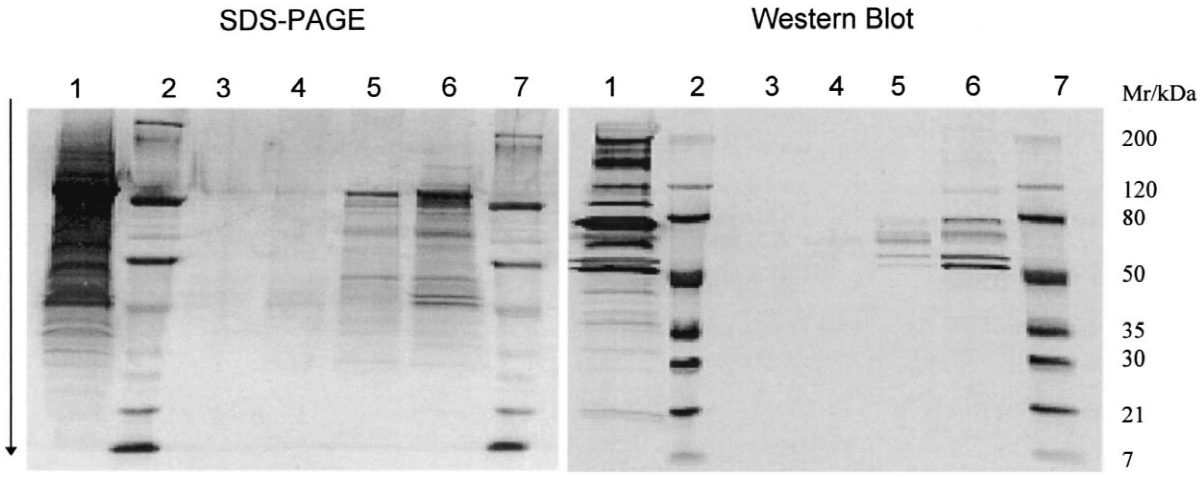

Fig. 5. SDS–PAGE and Western Blot of eluates collected from the effluent of affinity chromatography runs. Lane 1: vWF free pdFVIII concentrate Octavi SDP; lanes 2 and 7: broad-range-molecular-mass marker; lane 3: eluate from peptide 010; lane 4: eluate from peptide 011; lane 5: eluate from peptide 014; lane 6: eluate from peptide 015. For blotting experiments a 1:1:1:1 mixture of 1:1000 dilutions of antibodies 530, 531, 532 and 038 was used.

the various test formats. Two out of four could also retain pdFVIII when the peptides were immobilized onto chromatographic supports.

4. Discussion

By screening octapeptides derived from the interacting region of vWF in the vWF–FVIII complex, a large number of peptides with affinity for FVIII was identified.

These peptides were not in sequence and several areas reacted with FVIII, similar to the reactions of a non-linear epitope. These regions may be in close proximity in the vWF–FVIII complex. Since the three-dimensional (3D) structure of this complex is not yet available, a further interpretation of how these binding regions interact with FVIII is presently not possible. The identification of peptides with affinity for FVIII by the applied methodology could be expected, since the mapping of protein domains and epitopes utilizes the same strategy. The question was whether these peptides would also work as affinity ligands under chromatographic conditions.

Interestingly, the incubation of the generated peptide ladder with vWF-free pdFVIII showed less intensive spots than the incubation with rFVIII. The association of FVIII with vWF is very strong, thus we assume that the apparently vWF-free pdFVIII preparation was not completely free of vWF. Small vWF fragments which still bound to FVIII, may distort the interactions with certain peptides. Therefore, only peptides with affinity for pdFVIII and rFVIII were selected for further characterization. These peptides should be capable of binding FVIII independently from its source.

Longer peptides such as those used for the initial screening were constructed and their affinity for FVIII was measured with real-time interaction assays. Then their performance in affinity chromatography of FVIII was tested. Surprisingly, the peptide denoted as 010 showed a high affinity for FVIII in real-time interaction assays, but did not bind FVIII under chromatographic conditions when immobilized onto a chromatographic gel. Binding properties measured by real-time interaction assays for the peptide denoted as 014 were confirmed with affinity chromatography experiments. Independently of the test system, peptide 014 showed a strong affinity for both rFVIII and vWF-free pdFVIII. Band patterns of

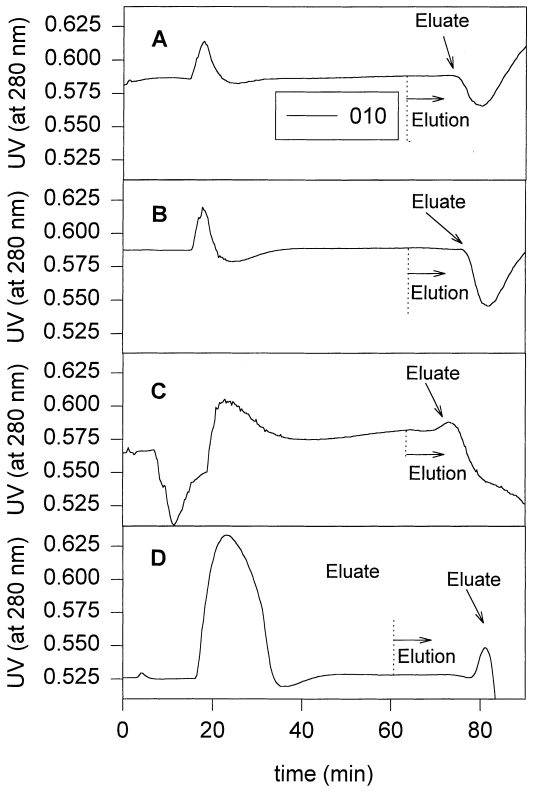

Fig. 6. Affinity chromatography with candidates immobilized onto epoxy Sepharose FF loading rFVIII. The rFVIII was loaded in 10 m*M* HEPES, pH 7.4 with 5 m*M* $CaCl_2$ and 0.1% (v/v) Triton X-100. (A) Peptide 010 (LCPPGMVRHE), (B) peptide 011 (RCPCFHQGK), (C) peptide 014 (CFHQGKEYA) and (D) peptide 015 (RDRKWNCTDHVC).

eluates from affinity chromatography with peptides 014 and 015 were different dependent on the source of FVIII. With rFVIII a clear band at 80 000 and at ≈200 000 were observed. These bands are interpreted as the light chain of FVIII and the complete molecule. The rFVIII preparation contained free light chain. With pdFVIII, the whole FVIII molecule and the light chain together with degradation products could be found in the eluates. The peptides seem to be directed against the light chain. They are able to pull out intact FVIII, the light chain, and their related degradation products. This is an explanation why the eluate composition varies with the source of FVIII.

Peptide 011 did not bind vWF-free pdFVIII in affinity chromatography experiments, but bound rFVIII under the same conditions. Peptide 015 turned out to be a valuable ligand for affinity chromatography, although real-time interaction assays indicated that candidates 010 and 014 showed a higher maximal response.

The contradiction between real-time interaction assays and affinity chromatography may have various reasons. One possible explanation could be the ligand presentation and ligand density on the surface. Different matrices, spacers and immobilization chemistries were used in both systems. The epoxy pre-activated Sepharose had 19–40 μ*M* active groups per ml drained gel (according to data provided by the manufacturer). By NHS/EDC-mediated activation, more functional groups may be introduced to the dextran-coated sensor chip in comparison to the Sepharose. Ligand density, apparent affinity and adsorption behavior are highly interrelated. The spots on the cellulose exhibit the highest ligand density – up to 10 m*M* (as described by Frank [27]). Peptide-carrying spots showed the highest apparent affinity, which is consistent with the relation between ligand density and adsorption capacity. Further work will be necessary to determine adsorption isotherms and binding kinetics. Biotinylated peptides showed a reaction with FVIII similar to that of a well-characterized monoclonal antibody directed against the light chain. The affinity for this region could be expected since the peptides were derived from the FVIII-binding region of vWF, which is located on the light chain. Competitive assays are not conclusive since the peptides are directed against different regions than those recognized by the antibodies used in our assays.

Such fluorescent or radioactively labeled peptides could also be used for in situ labeling of FVIII. After incubation of the labeled peptide with FVIII, the peptide–FVIII complex could be separated by chromatographic or electrophoretic techniques. For static methods the peptides could be conjugated with biotin or an enzyme, for instance. These labeled peptides could be used for quantitative or qualitative determination of FVIII in blotting techniques, such as dot-blot or Western blots.

We consider peptides as tools for static blotting techniques, for in situ labeling of FVIII followed by

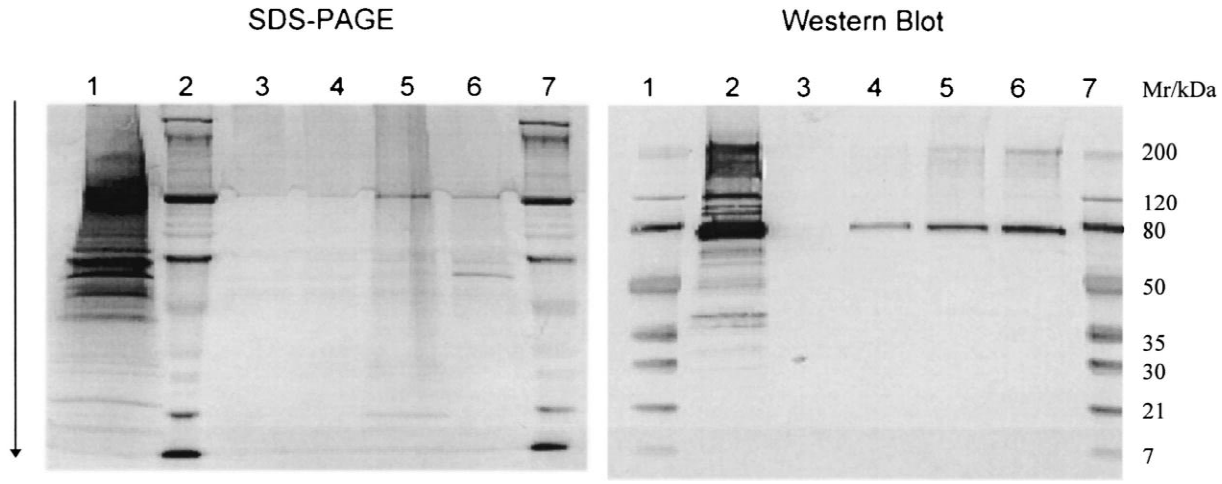

Fig. 7. SDS–PAGE and Western Blot of eluates collected from the effluent of affinity chromatography runs with rFVIII. Lanes 2 and 7: broad-range-molecular-mass marker; lane 1: rFVIII; lane 3: eluate from peptide 010; lane 4: eluate from peptide 011; lane 5: eluate from peptide 014; lane 6: eluate from peptide 015. For blotting experiments a 1:1:1:1 mixture of 1:1000 dilutions of antibodies 530, 531, 532 and 038 was used.

separation with capillary electrophoresis, and as ligands for affinity chromatography.

Further work is planned in which we will describe the performance and characteristics of the candidates such as capacity, electrophoretic mobility, solubility and binding kinetics.

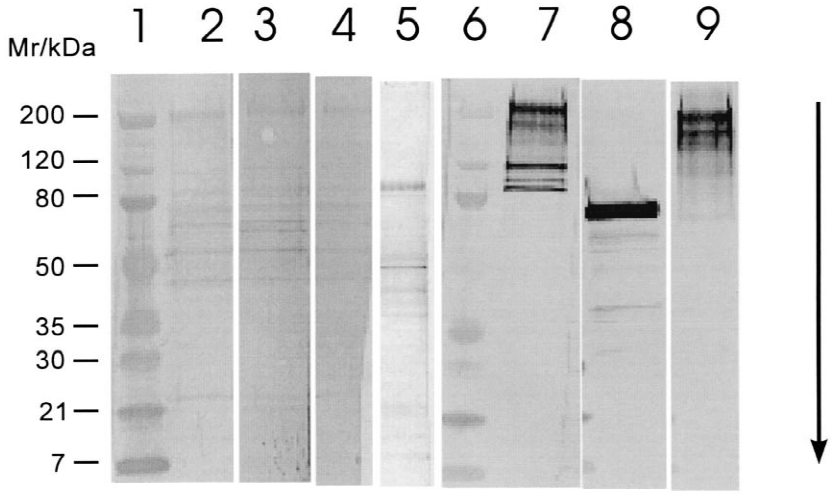

Fig. 8. Determination of the FVIII-binding region of candidates. The rFVIII was electrophoretically separated and electroblotted onto a nitrocellulose membrane. The biotinylated candidates were added and incubation with streptavidin–alkaline phosphatase-conjugate was followed by the substrate NBT–BCIP. Bound biotinylated peptides were identified as blue stained bands. Lane 1: broad-range-molecular mass marker; lane 2: peptide 010; lane 3: peptide 011; lane 4: peptide 014; lane 5: peptide 015; lane 6: broad-range-molecular-mass marker; lane 7: mAb 530p; lane 8: mAb 038; and lane 9: mAb 532p.

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