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Sequence location of a putative transglutaminase cross-linking site in human vitronectin

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We attempted to locate the glutamine residue in human vitronectin, susceptible to cross-linking by transglutaminases. Vitronectin was incubated with ^{14}C-labelled putrescine and plasma factor XIIIa and, after reduction and alkylation, the vitronectin was digested with trypsin. HPLC of the digest followed by scintillation counting revealed one major and two minor radioactivity labelled peaks. Sub-digestion with *Staphylococcus aureus* protease, sequence analysis and mass-spectrometry of the resulting peptides demonstrated that Gln-93 of vitronectin had incorporated putrescine. Additionally, Gln-73, Gln-84 and Gln-86 were found to be minor sites for incorporation.

Vitronectin; Serum spreading factor; S-protein; Transglutaminase; Factor XIIIa; Cross-linking

1. INTRODUCTION

Vitronectin (VN) is a multifunctional glycoprotein present in human blood plasma at a concentration of ~ 250 μg/ml [1]. In its 75-kDa single-chain form it is sensitive to proteolysis in the circulation. Therefore, part of the VN in plasma is in a two-chain form, consisting of a 65-kDa chain disulphide-bonded to a 10-kDa fragment [2]. VN has been purified in several laboratories [3-6]. The complete amino-acid sequence (459 residues) deduced from the cDNA sequence [7,8] reveals that somatomedin B constitutes the 44 amino-terminal residues of VN [9]. These are followed immediately by an Arg-Gly-Asp sequence, which is part of the recognition site for the VN receptor [10]. The binding of VN to its receptor mediates the attachment and spreading of fibroblasts and epithelial cells [10,11], whence the name serum spreading factor is derived. VN inhibits the formation of the complement membrane attack complex through binding to the C5b-7 complex, thereby preventing the polymerization of C9 and its insertion into membranes [3,12]. In addition, VN binds to the [thrombin:antithrombin III] complex and inhibits the heparin-dependent formation thereof [13-16]. VN also binds plasminogen activator inhibitor 1 (PAI-1) in plasma [17,18].

Recently, it was shown that VN is a substrate for transglutaminases [19], and here we report the localization of the glutamine residue to which putrescine is cross-linked by FXIIIa-catalysed transamidation.

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Abbreviations: VN, vitronectin; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; PDMS, plasma desorption mass-spectrometry; FXIII, plasma factor XIII; putrescine, 1,4-diamino butane; *S. aureus* protease, *Staphyloccocus aureus* V8 protease; a.m.u., atomic mass units

2. MATERIALS AND METHODS

VN was isolated from human plasma according to Dahlbäck and Podack [5] and frozen in the final buffer (0.01 M Tris, 0.15 M NaCl, pH 7.4; 395 μg/ml). FXIII was isolated from bovine plasma as described in reference [20] and purified further on Protein A-Sepharose (Pharmacia, Uppsala). Thrombin was a kind gift from Dr. J.W. Fenton II (State Department of Health, Albany, NY).

In the labelling experiment, 3 mg VN was thawed and made 15 mM in $CaCl_2$. 3 mg putrescine (Sigma, St. Louis, MO) containing 25 μCi [1,4-^{14}C)]putrescine (109 mCi/mmol, Amersham) was added. 0.5 mg FXIII (1 mg/ml) was activated (1 h, 37°C) with 10 μg thrombin (4000 U/mg) and added to the VN/putrescine solution. The reaction mixture (vol. 10.5 ml) was incubated for 4 h at 37°C.

Reduction and carboxymethylation of VN was performed directly in the VN/FXIIIa/putrescine reaction mixture as follows: urea, Tris and EDTA were added so as to give a final concentration of 8 M urea, 0.2 M Tris and 20 mM EDTA (final volume 20 ml, pH 8.2). 3 mg dithioerythritol (Merck, Darmstadt) was added and the mixture was incubated for 1 h at 20°C. A surplus of iodoacetic acid (Merck, Darmstadt) was then added and the mixture was incubated for 1/2 h at 20°C.

Desalting of the resulting solution was performed on a column of Sephadex G-25 (Pharmacia, Uppsala; 2 × 60 cm) in 0.1 M ammonium bicarbonate. The protein peak (20 ml) was digested by direct addition of 100 μg trypsin (Worthington, Freehold, NJ) (22 h, 37°C). Soybean trypsin inhibitor (Sigma) was added and the solution was freeze-dried.

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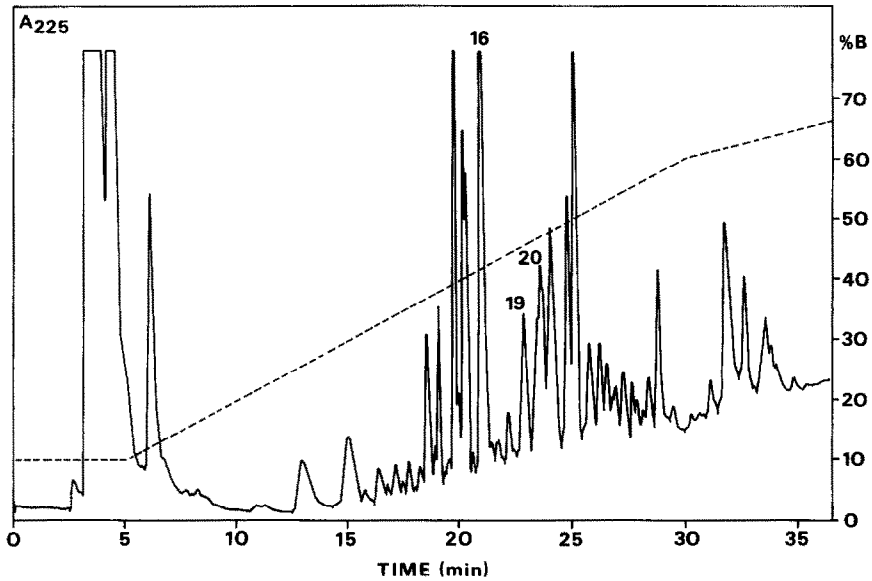

Fig.1. HPLC separation of peptides obtained from VN by digestion with trypsin. A Nucleosil C_{18} 7 μ column (0.4 × 24 cm) was used, eluted with a three-step linear gradient of 96% ethanol (B) in 0.1% trifluoroacetic acid (A). The flow rate was 1 ml/min. (——) A_{225}nm (arbitrary units); (– – –) %B. Peaks 16, 19 and 20 were radioactive.

Separation of fragments and peptides was carried out by reverse-phase HPLC with a Hewlett Packard 1084B liquid chromatograph, and aliquots from each peak were counted in a scintillation counter (Beckman LS 1801).

Peptide hydrolysates were analysed in a Hewlett Packard Amino Quant analyzer. Amino-acid-sequencing was performed by automated Edman degradation with an Applied Biosystems 477A or 470A gas-phase amino-acid sequencer, and PTH derivatives were identified by reverse-phase HPLC by using an Applied Biosystems Model 120A PTH amino acid analyzer or a Hewlett Packard 1084B liquid chromatograph.

Mass-spectrometry was performed by using a BioIon 10K plasma desorption time-of-flight instrument. Samples were dissolved in 0.1% trifluoroacetic acid and applied to nitrocellulose-covered targets, spin-dried and micro-rinsed as described [21]. Spectra were recorded at 16 kV for 10^6 primary fission events.

Standard chemicals were from Merck, Sigma or Fluka (Buchs). *S. aureus* protease was from Worhtington.

3. RESULTS AND DISCUSSION

One major and two minor radioactively labelled peaks were found in the tryptic digest of the ^{14}C-labelled VN (fig.1). The major peak (peak 20) was sequenced and the N-terminal sequence was GNPE-QTPVLKPEEEA, corresponding to residues 89–103 of VN. Scintillation counting of the PTH amino acids showed radioactivity in cycle 5. To make certain that this was the only labelled glutamine in the major radioactive peak, the fragment was sub-digested with *S. aureus* protease. The resulting peptides were separated by HPLC (fig.2) and aliquots were scintillation counted. Only one radioactive peak was found (peak 14), and sequencing of this peptide gave the amino acid sequence QTPVLKPEEEAPAPE (VN 93–107). The yield of the glutamine in cycle one was very low (7 pmol) compared with the yield in other cycles (169, 152 and 158 pmol in cycles 3 (Pro),4 (Val) and 5 (Leu), respectively) (table 1), indicating that the putrescine moiety was linked to this Gln-1. Scintillation counting of the PTH amino acids confirmed this, showing radioactivity in cycle 1 (table 1). As a final confirmation, the peptide was analysed by mass spectrometry. This showed the mass of the intact molecule to fit the mass calculated for the peptide including putrescine (fig.3B). The same peptide, without an attached putrescine moiety, was identified in a neighbouring

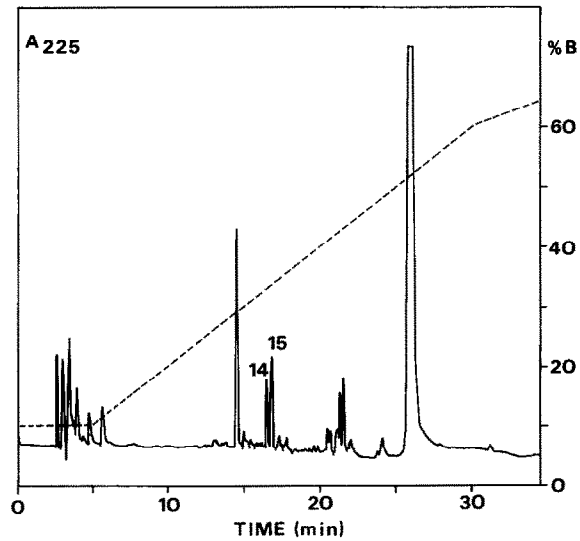

Fig.2. HPLC-purification of peptides obtained from peak 20 (in fig.1) by digestion with *S. aureus* protease. A Nucleosil C_{18} 7μ column (0.4 × 24 cm) was used, eluted with a three-step linear gradient of 80% acetonitrile, 0.02% trifluoroacetic acid (B) in 0.1% trifluoroacetic acid (A). The flow rate was 1 ml/min. (——) A_{225}nm (arbitrary units); (– – –) %B. Peak 14 was radioactive.

Table 1

Sequence of the [^{14}C]putrescine labelled *S. aureus* protease peptide, VN (93–107), 240 pmol, from peak 15 (fig.2)

Cycle	Position	Known sequence	Found sequence	Pmol	Radioactivity cpm
1	93	Gln	(Gln)	7	79
2	94	Thr	Thr	94	47
3	95	Pro	Pro	169	45
4	96	Val	Val	152	37
5	97	Leu	Leu	158	35
6	98	Lys	Lys	113	39
7	99	Pro	Pro	148	39
8	100	Glu	Glu	59	35
9	101	Glu	Glu	70	37
10	102	Glu	Glu	84	40
11	103	Ala	Ala	95	44
12	104	Pro	Pro	93	40
13	105	Ala	Ala	94	49
14	106	Pro	Pro	89	36
15	107	Glu	(Glu)	13	39

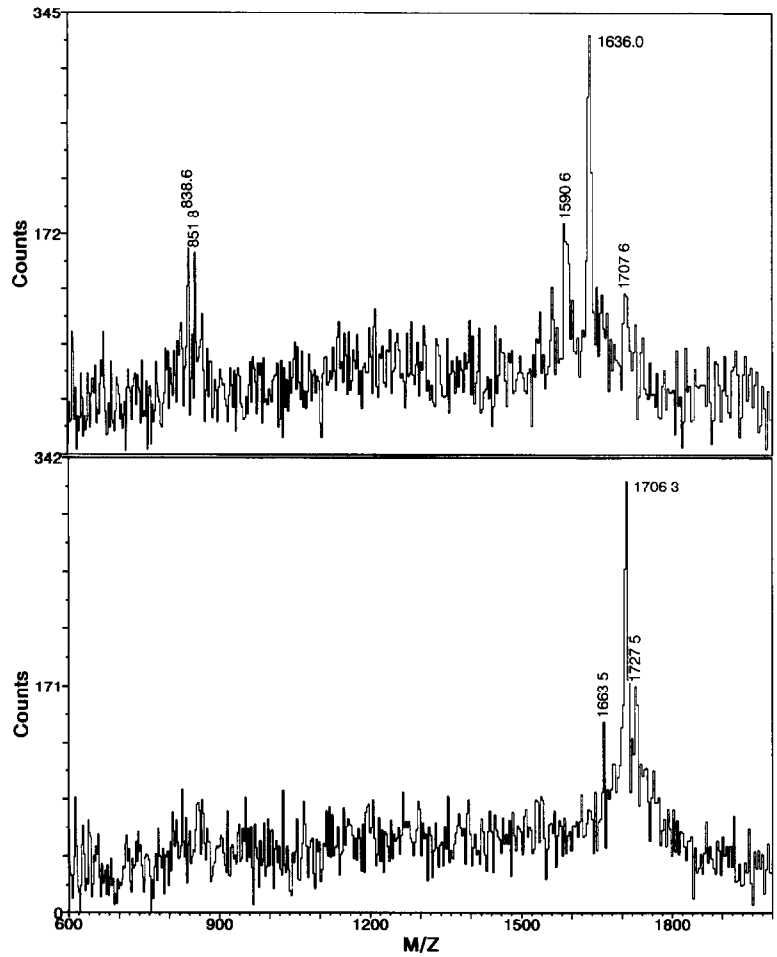

Fig.3. (A) PDMS spectrum of fraction 15 (fig.2), showing the MH^+ ion (m/z 1636.0) of unmodified VN (93–107) peptide. A small amount of the same peptide with putrescine attached is present (MH^+ ion of m/z 1707.6). The peaks at 838.6 and 851.8 arise from an unidentified impurity. (B) PDMS spectrum of fraction 14 (fig.2), showing the MH^+ ion of the putrescine linked VN (93–107) peptide (m/z 1706.3). The peaks at m/z 1590.6 (A) and m/z 1663.5 (B) arise from loss of CO_2 and the m/z 1727.5 peak (A) is a sodium adduct ion.

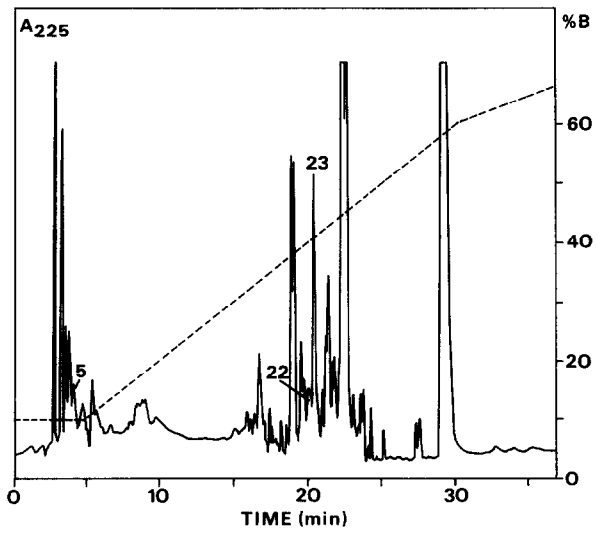

Fig.4. HPLC-purification of peptides obtained from peak 16 (in fig.1) by digestion with *S. aureus* protease. The column and gradient used were the same as in fig.2. Peaks 5, 22 and 23 were radioactive.

fraction, containing slightly more protein (peak 15, fig.2) (fig.3A).

The two minor radioactively labelled peaks (peaks 16 and 19, fig.1) were analyzed in the same way as peak 20. The N-terminal sequence of peak 19 was the same as that of peak 20, except that an additional sequence, GQYXYELDEK, corresponding to residues 158–167 of VN, was found. This could be explained by a disulfide bridge between Cys-137 and Cys-161, which was still intact, despite the reduction and alkylation. Digestion with *S. aureus* protease and separation of peptides by HPLC (not shown), under the same conditions as in fig.2, revealed a radioactive peptide identical to that found in peak 14, fig.2. In addition, the presence of a disulfide bridge between Cys-137 and Cys-161 was confirmed by the isolation of the peptide

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137
LCSGKPFDAFTDLK
  |
GQYCYE
  161
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The N-terminal sequence of peak 16 showed a mixture of two sequences, DQESXKGRXTE (residues 1–11 of VN) and GDVFTMPEDE (residues 46–55 of VN), and none of the resulting PTH amino acids were radioactive. The fragment mixture was then digested with *S. aureus* protease and the resulting peptides were separated by HPLC (fig.4). Three radioactively labelled peaks were identified (peaks 5, 22 and 23). Sequencing of these gave LQAQSK for peak 5 and QVGGPSLTSDLQAQSK for peaks 22 and 23, corresponding to residues 83–88 and 73–88 of VN. These peptides are obviously cleavage variants, originating from an incomplete cleavage by *S. aureus* protease at Asp-82. The yields of Gln-73, Gln-84 and Gln-86 were relatively low and scintillation counting of the PTH amino acids gave weak indication of radioactivity in Gln-73 and Gln-86 (table 2). Mass spectrometry of peptide VN 83-88 showed a molecular mass that indicated the presence of a single putrescine unit (fig.5). Mass spectrometry of the VN 73-88 peptide from peak 22 showed the presence of two peptides with masses corresponding to attachment of one and two putrescine units, respectively (fig.6B). The neighbouring peak in the HPLC chromatogram (peak 23) contained the identical peptide without putrescine, but also a small amount of the peptide with one unit attached (fig.6A).

The results shown in this paper demonstrate clearly that VN is a substrate for FXIIIa and that putrescine

Table 2

Sequence of the [^{14}C]putrescine labelled *S. aureus* protease peptides, VN (73–88), 100 pmol and VN (83–88), 170 pmol, from peaks 23 and 5 (fig.4)

Cycle	Position	Known sequence	Found sequence	Pmol		Radioactivity cpm	
1	73	Gln	Gln	29		48	
2	74	Val	Val	64		36	
3	75	Gly	Gly	70		34	
4	76	Gly	Gly	73		37	
5	77	Pro	Pro	53		33	
6	78	Ser	Ser	45		33	
7	79	Leu	Leu	47		36	
8	80	Thr	Thr	36		29	
9	81	Ser	Ser	26		28	
10	82	Asp	Asp	13		36	
11	83	Leu	Leu	23	156	31	33
12	84	Gln	Gln	6	68	38	34
13	85	Ala	Ala	27	117	39	30
14	86	Gln	Gln	9	48	35	46
15	87	Ser	Ser	–	47	–	34
16	88	Lys	Lys	–	26	–	34

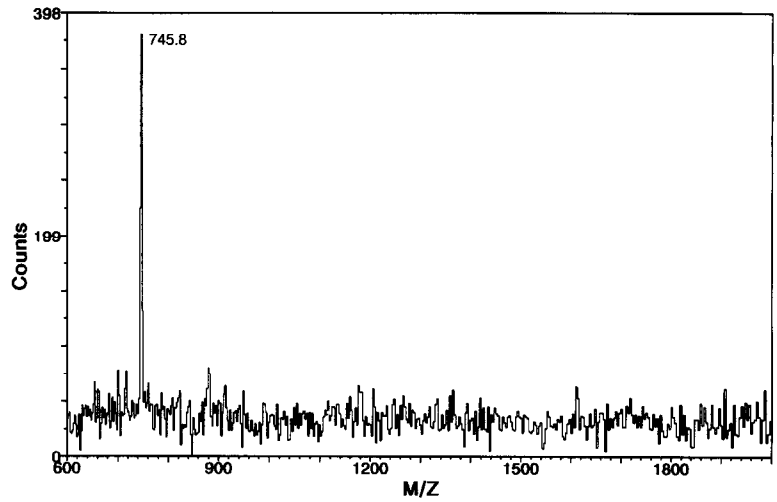

Fig.5. PDMS spectrum of peak 5 (fig.4). The MH^+ ion at m/z 745.8 corresponds to the VN 83-88 peptide with one putrescine unit attached.

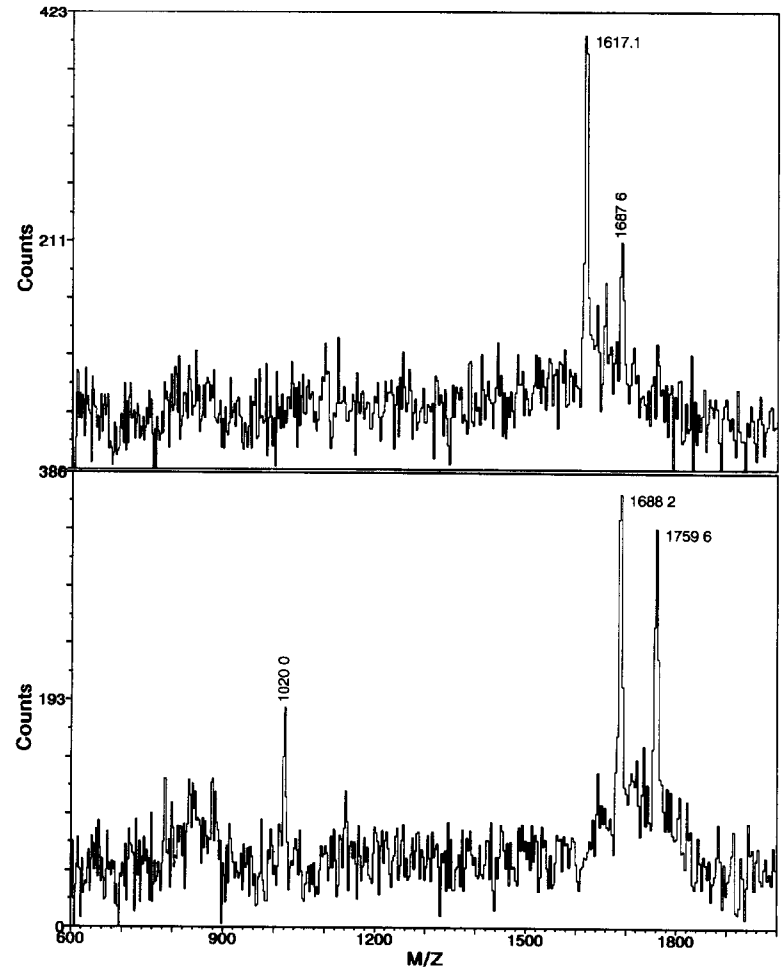

Fig.6. (A) PDMS spectrum of peak 23 (fig.4). The MH^+ ion at m/z 1617.1 corresponds to the VN 73-88 peptide and the MH^+ ion at m/z 1687.6 is the same peptide with one putrescine unit attached. (B) PDMS spectrum of peak 22 (fig.4). This fraction contains the same peptide as spectrum A, but with one (m/z 1688.2) or two (m/z 1759.6) putrescine units attached.

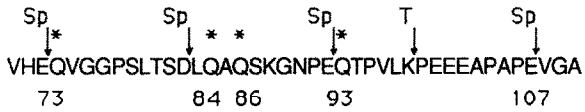

Fig.7. Amino-acid sequence around the putative FXIIIa cross-linking site(s) in VN (*). The sequence is from [7,8]. The cleavage sites for trypsin (T) and *S. aureus* protease (Sp) are indicated. Gln-93 is the major cross-linking site, while Gln-73, Gln-84 and Gln-86 are minor sites.

can be linked to Gln-93 of VN (fig.7). Additionally, at least two out of three other glutamine residues nearby (Gln-73, Gln-84 and Gln-86) are also susceptible to transamidation catalysed by FXIIIa. The cross-linking sites are located in a negatively charged connecting strand (residues 45–131) between the somatomedin B domain [22] and the first of two hemopexin domains [23]. The connecting strand in addition contains the RGD cell adhesion sequence, a potential *N*-glycosylation site [7,8] and two putative tyrosine sulfation sites [24].

As an additional finding, a disulfide bridge connecting Cys-137 to Cys-161, was identified.

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REFERENCES

[1] Shaffer, M.C., Foley, T.P. and Barnes, D.W. (1984) J. Lab. Clin. Med. 103, 783–791.
[2] Tomasini, B.R. and Mosher, D.F. (1986) Blood 68, 737–742.
[3] Podack, E.R. and Müller-Eberhard, H.J. (1979) J. Biol. Chem. 254, 9908–9914.
[4] Barnes, D.W. and Silnutzer, J. (1983) J. Biol. Chem. 258, 12548–12552.
[5] Dahlbäck, B. and Podack, E.R. (1985) Biochemistry 24, 2368–2374.
[6] Preissner, K.T., Wassmuth, R. and Müller-Berghaus, G. (1985) Biochem. J. 231, 349–355.
[7] Jenne, D. and Stanley, K.K. (1985) EMBO J. 4, 3153–3157.
[8] Suzuki, S., Oldberg, Å., Hayman, E.G., Pierschbacher, M.D. and Ruoslahti, E. (1985) EMBO J. 4, 2519–2524.
[9] Barnes, D.W., Foley, T., Jr., Shaffer, M.C. and Silnutzer, J.E. (1984) J. Clin. Endocrinol. Metab. 59, 1019–1021.
[10] Pytela, R., Pierschbacher, M.D. and Ruoslahti, E. (1985) Proc. Natl. Acad. Sci. USA 82, 5766–5770.
[11] Suzuki, S., Argraves, W.S., Pytela, R., Arai, H., Kruslus, T., Pierschbacher, M.D. and Ruoslahti, E. (1986) Proc. Natl. Acad. Sci. USA 83, 8614–8618.
[12] Podack, E.R. and Müller-Eberhard, H.J. (1980) J. Immunol. 124, 1779–1783.
[13] Ill, C.R. and Ruoslahti, E. (1985) J. Biol. Chem. 260, 15610–15615.
[14] Jenne, D., Hugo, F. and Bhakdi, S. (1985) Thromb. Res. 38, 401–412.
[15] Podack, E.R., Dahlbäck, B. and Griffin, J.H. (1986) J. Biol. Chem. 261, 7387–7392.
[16] Preissner, K.T. and Müller-Berghaus, G. (1987) J. Biol. Chem. 262, 12247–12253.
[17] Declerck, P.J., Mol, M.D., Alessi, M.-C., Baudner, S., Pâques, E.-P., Preissner, K.T., Müller-Berghaus, G. and Collen, D. (1988) J. Biol. Chem. 263, 15454–15461.
[18] Wiman, B., Almquist, Å., Sigurdardottir, O. and Lindahl, T. (1988) FEBS Lett. 242, 125–128.
[19] Sane, D.C., Moser, T.L., Pippen, A.M.M., Parker, C.J., Achyuthan, K.E. and Greenberg, C.S. (1988) Biochem. Biophys. Res. Commun. 157, 115–120.
[20] Halkier, T. and Magnusson, S. (1988) Thromb. Res. 51, 313–324.
[21] Nielsen, P.E., Klarskov, K., Højrup, P. and Roepstorff, P. (1988) Biomed. Environm. Mass Spectrom. 17, 355–362.
[22] Patthy, L. (1988) J. Mol. Biol. 202, 689–696.
[23] Jenne, D. and Stanley, K.K. (1987) Biochemistry 26, 6735–6742.
[24] Jenne, D., Hille, A., Stanley, K.K. and Huttner, W.B. (1989) Eur. J. Biochem. 185, 391–395.