The heparin-binding site in tetranectin is located in the N-terminal region and binding does not involve the carbohydrate recognition domain

Rikke H. LORENTSEN, Jonas H. GRAVERSEN, Nigel R. CATERER, Hans C. THØGERSEN and Michael ETZERODT[1]

Laboratory of Gene Expression, Department of Molecular and Structural Biology, University of Aarhus, Gustav Wieds Vej 10, DK-8000 Aarhus C, Denmark

Tetranectin is a homotrimeric plasma and extracellular-matrix protein that binds plasminogen and complex sulphated polysaccharides including heparin. In terms of primary and tertiary structure, tetranectin is related to the collectin family of Ca^{2+}-binding C-type lectins. Tetranectin is encoded in three exons. Exon 3 encodes the carbohydrate recognition domain, which binds to kringle 4 in plasminogen at low levels of Ca^{2+}. Exon 2 encodes an α-helix, which is necessary and sufficient to govern the trimerization of tetranectin by assembling into a triple-helical coiled-coil structural element. Here we show that the heparin-binding site in tetranectin resides not in the carbohydrate recognition domain but within the N-terminal region, comprising the 16 amino acid residues encoded by exon 1. In particular, the lysine residues in the decapeptide segment KPKKIVNAKK (tetranectin residues 6–15) are shown to be of primary importance in heparin binding.

Key words: C-type lectin, proteoglycan, refolding.

INTRODUCTION

Tetranectin (TN) is a homotrimeric [1] Ca^{2+}- and plasminogen (Plg)-binding protein found in plasma [2] and as a component of the extracellular matrix during muscle development and regeneration [3]. TN has been proposed to have a role in mineralization during osteogenesis [4,5]. Furthermore, TN has been found deposited in the stromas of some human carcinomas, whereas little or no TN is found in the corresponding normal tissues [6–8]. TN and Plg are co-localized at the invasive front of cutaneous melanoma lesions [9]. Collectively, these investigations are suggestive of a physiological role for TN in development and tissue remodelling.

TN binds specifically to Plg kringle 4 [2] and apolipoprotein (*a*) [10] in a lysine-sensitive way, and, dependent on Ca^{2+}, to fibrin [11]. The mature TN monomer, of 181 amino acid residues and containing three internal disulphide bridges, is encoded by three exons [8,12], of which the polypeptide encoded by exon 2 is necessary and sufficient for trimerization [1]. The Plg kringle-4-binding site is located within the domain encoded by exon 3 [13], which encodes a domain similar with the carbohydrate recognition domains (CRDs) of the C-type lectin superfamily [14]. TN exhibits pronounced structural similarity to both the neck region and the CRD of the collectins, and binds Ca^{2+} in a similar way [15,16]. The binding to Plg kringle 4 has been shown to involve amino acid residues near and within the Ca^{2+}-binding site 2; accordingly, Plg kringle 4 and Ca^{2+} are competitive ligands for the CRD domain of TN [13]. No carbohydrate ligand for the Ca^{2+}-saturated TN CRD has yet been identified.

TN isolated from human plasma (native TN, nTN) has been reported to bind sulphated glycosaminoglycans including heparin, heparan sulphate, fucoidan and chondroitin sulphates A, B and C; these interactions were proposed to depend mainly on the sulphate groups of the saccharides [17]. Furthermore, nTN is modified post-translationally on Thr-4 [18]; the modification has been identified as an O-linked oligosaccharide with the covalent structure *N*-acetylhexosamine-[hexose, $(\text{sialic acid})_{0-3}$] [19].

Heparin is a sulphated glycosaminoglycan, primarily composed of repeated disaccharide units of iduronic acid and glucosamine. Sulphated glycosaminoglycans are known to bind a variety of proteins and to have a role in many physiological processes, including smooth-muscle cell proliferation, nerve cell development, tumour growth, angiogenesis and metastasis [20]. Apart from the occurrence of basic clusters in the heparin-binding regions, with spatial separations of the basic residues of approx. 20 Å, no single unifying heparin-binding motif has yet been established [21,22]. In line with the prominent role of basic clusters in the binding to the negatively charged heparin, the interactions have been described as being based mainly on ion-exchange effects. Linear relationships between $\log(K_d)$ and log(ionic strength) have been established for several heparin-binding proteins, including fibroblast growth factor [23], thrombin, anti-thrombin [24,25], mucus proteinase inhibitor [26] and lipoprotein lipase [27]. Furthermore, basic fibroblast growth factor exhibited a linear dependence between the NaCl concentration at which basic fibroblast growth factor and single or double alanine mutants eluted from a heparin-Sepharose column, and the $\log(K_d)$ for the binding of these proteins to low-molecular-mass heparin in solution [23].

In the present study we examined deletion variants of TN corresponding to exon boundaries to locate the heparin-binding site in TN and investigate the Ca^{2+} sensitivity of the interaction by heparin-Sepharose affinity chromatography. The heparin-binding site was further characterized by analysing the single-residue substitution of lysine residues in the region with alanine and a chimaeric construct in which residues 1–26 of human TN were replaced with the corresponding residues from murine TN.

EXPERIMENTAL

nTN

nTN was purified from human plasma essentially as described [18].

Abbreviations used: CRD, carbohydrate recognition domain; NTA, nitrilotriacetic acid; TN, tetranectin; nTN, tetranectin isolated from human plasma; Plg, plasminogen; rTN, recombinant TN; $[rTN_f]_i$, concentration of free rTN; V_e, elution volume (elution zone maximum).

[1] To whom correspondence should be addressed (e-mail met@biobase.dk).

Construction, expression and processing of recombinant TN derivatives

The recombinant TN (rTN) variants studied were identical with those described in [1], except that the construct representing the polypeptide encoded by exons 1 and 2 (rTRIP-A) corresponded to amino acid residues 1–54 and with Cys-50 replaced by Ser, whereas the rTN12 derivative described earlier [1] encoded residues 1–49. rTN3 corresponded to residues 45–181 (exon 3), encoding the CRD of TN. rTN23 corresponded to residues 17–181 (exons 2 and 3). Single-residue mutations in rTN were performed with the Quickchange kit (Stratagene, La Jolla, CA, U.S.A.) in accordance with the manufacturer's recommendations. pT7H6FX-rTN123 was used as template for mutagenesis. Mutagenesis primers were purchased from DNA Technology (Aarhus, Denmark). The initial melting temperatures for all primer pairs were at least 62 °C, with the mutation sites located centrally. The chimaeric rM1TN construct was prepared by replacing the human TN sequence from Glu-1 to Leu-26 with the corresponding murine TN sequence [28], using the shared *Sac*I restriction enzyme site (see Figure 4). Expression in *Escherichia coli* and purification of the unfolded rTN fusion protein derivatives were performed as described [1]. Refolding of the rTN, rTN23, rTN3 and rM1TN derivatives were performed by loading the fusion proteins on Ni^{2+}-charged nitrilotriacetic acid (NTA) columns in 8 M urea/0.5 M NaCl/50 mM Tris/HCl (pH 8)/10 mM 2-mercaptoethanol. After being loaded, the columns were subjected to a cyclic refolding procedure [29] in which the renaturation buffer, buffer A [0.5 M NaCl/50 mM Tris/HCl (pH 8)/2 mM $CaCl_2$/2 mM reduced glutathione/0.2 mM oxidized glutathione], was passed over the column at 2 ml/min. Every 45 min a pulse, with a duration of 15 min, of denaturing buffer [buffer B; 100 % B being 8 M urea/0.5 M NaCl/50 mM Tris/HCl (pH 8)/2 mM $CaCl_2$/3 mM reduced glutathione] was passed over the column at 2 ml/min. In each consecutive cycle the percentage of buffer B was decreased by 4 % with buffer A, from an initial 100 % to 20 %. Refolded fusion protein derivatives were eluted from the columns by EDTA [500 mM NaCl/50 mM Tris/HCl (pH 8)/10 mM EDTA] and the fusion proteins were cleaved with bovine blood coagulation factor X_a (Protein Engineering Technology, Aarhus, Denmark) at 1:200 (w/w) at 4 °C overnight. The eluted rTN and rM1TN derivatives were taken into a 50 mM NaCl/25 mM sodium acetate buffer (pH 5) by gel filtration on Sephadex G25 and loaded on 50 ml SP-Sepharose columns (Pharmacia-Amersham) and gradient-eluted from 50 mM to 1 M NaCl over 20 column vol. Fractions containing cleaved and monomeric protein, as judged by non-reducing SDS/PAGE analysis, were pooled and the buffer was exchanged to 20 mM NaCl/50 mM Tris/HCl (pH 8) by gel filtration on Sephadex G25 before being loaded on 20 ml Q-Sepharose columns (Pharmacia-Amersham) and gradient-eluted from 20 to 500 mM NaCl over 50 column vol. The rTN23 and rTN3 derivatives eluted from the Ni^{2+}-charged NTA columns were gel-filtered into buffer containing 20 mM NaCl and 50 mM Tris/HCl, pH 8, and loaded on 20 ml Q-Sepharose columns.

Fractions containing essentially pure monomeric protein, as judged by non-reducing SDS/PAGE analysis, were pooled and concentrated by ultrafiltration (Amicon) and the buffer was exchanged to 100 mM NaCl/50 mM Tris/HCl (pH 8) by gel filtration on Sephadex G25.

The rTRIP-A fusion protein was refolded on the Ni^{2+}-charged NTA column by removal of the denaturant and 2-mercaptoethanol in a gradient over 1 column vol. into 0.5 M NaCl/50 mM Tris/HCl (pH 8). The refolded protein was eluted by 0.5 M NaCl/50 mM Tris/HCl (pH 8)/10 mM EDTA and cleaved with bovine blood coagulation factor X_a at 4 °C overnight (1:200, w/w). After being cleaved, the protein was taken into a buffer containing 50 mM NaCl and 25 mM sodium acetate, pH 5, and loaded on a 50 ml SP-Sepharose column and gradient-eluted from 50 mM to 1 M NaCl over 20 column vol. Fractions containing the cleaved and pure rTRIP-A protein, as judged by SDS/PAGE analysis, were pooled, concentrated by ultrafiltration and gel-filtered into 100 mM NaCl/50 mM Tris/HCl (pH 8).

Heparin-Sepharose chromatography

Protein sample (0.25 mg) was applied to 4 ml of heparin-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) in buffer C [100 mM NaCl/50 mM Tris/HCl (pH 8.0)], and gradient-eluted over 20 column vol. in a buffer containing 50 mM Tris/HCl, pH 8.0, and from 100–500 mM NaCl. After the gradient elution the heparin-Sepharose column was washed with 5 column vol. of 8 M urea/1 M NaCl/50 mM Tris/HCl (pH 8). Elution peaks in analytical chromatograms were fitted to Gaussian curves and the elution volume (V_e), defined as the elution zone maximum, and values of FWHM (full width at half maximum) were determined.

The apparent K'_a for the interaction between rTN and heparin was determined in a series of experiments with semi-quantitative affinity chromatography at room temperature, essentially as described [30]. Dry-cake heparin-Sepharose (20 mg), prewashed with buffer C, was taken into 300 μl of the same buffer (V_0). The heparin-Sepharose suspension was titrated by the stepwise addition of 40 μl (V) of 0.553 μM rTN (T_0) in buffer C. After each addition and mixing the mixture was left to equilibrate for 5 min and was subsequently centrifuged. The A_{280} of the cleared supernatant was measured to determine the concentration of free rTN ($[rTN_r]_i$).

In each series of experiments, $[rTN_r]_i$ was plotted against the number of additions (i) and the resulting curve was fitted to the expression:

$$i = \frac{V\{K'_a([rTN_r]_i + H_0) + 1\}}{V_0\{T_0([rTN_r]_i^{-1} + K'_a) - 1 - [rTN_r]_i K'_a\}}$$

where H_0 is the initial concentration of binding sites present on the heparin-Sepharose, and K'_a and H_0 were determined. This expression can be derived from the equlibrium equation:

$$K'_a = [rTN_r]_i[H]_i/[rTNH]_i$$

by substituting

$$[rTNH]_i = iVT_0/(iV + V_0) - [rTN_r]_i$$

and

$$[H]_i = V_0H_0/(iV + V_0) - [rTNH]_i$$

Determination of the apparent K'_a for the binding of the single-residue mutant rTN K9A was performed identically except that $T_0 = 0.325$ μM and $V = 26$ μl. Data analysis was performed with the program Origin™, version 4.1 (MicroCal Software, Northampton, MA, U.S.A.).

RESULTS AND DISCUSSION

Protein expression and refolding of rTN and mutants

Expression levels and refolding efficiency of the single-residue TN mutants and of the chimaeric construct were similar to those obtained for wild-type TN. The yields of the purified rTN derivatives were approx. 2 mg/l of culture, except for rTRIP-A and rTN23, which were 25 and 0.5 mg/l respectively. Purified

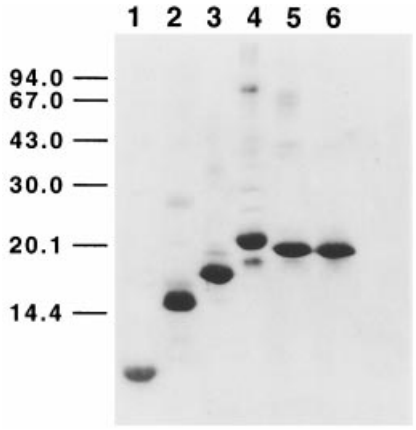

Figure 1 Non-reducing SDS/PAGE analysis of rTN, nTN and derivatives

Non-reducing SDS/PAGE analysis of representative samples of TN protein products. Lanes 1–6, rTRIP-A, rTN3, rTN23, nTN, rTN and rM1TN respectively. The positions and molecular masses (in kDa) of marker proteins are indicated at the left.

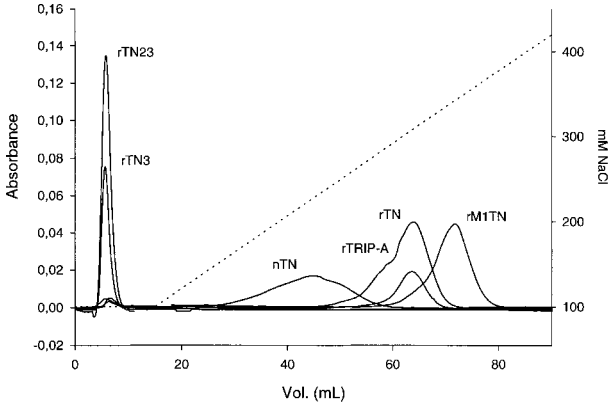

Figure 2 Heparin-Sepharose chromatography

Elution profiles (absorbance) of the proteins rTN, rTN23, rTN3, nTN, M1TN and rTRIP-A from heparin-Sepharose chromatograms. Absorbance was measured at 280 nm for all samples except rTRIP-A, which was monitored at 235 nm. The broken line represents the NaCl gradient.

protein preparations seemed essentially homogeneous and free from disulphide-linked oligomeric species as judged by non-reducing SDS/PAGE analysis (Figure 1). All the full-length single-residue mutants and the chimaeric construct exhibited wild-type Plg kringle 4 affinity (results not shown). SDS/PAGE staining intensities for rTN and mutants confirmed that the use of a rTN $A^{1\%}_{1\,cm,280}$ of 20 g·litre^{-1}·cm^{-1} [13] was justifiable for the determination of protein concentration. For rTRIP-A an $A^{1\%}_{1\,cm,235}$ of 8 g·litre^{-1}·cm^{-1} was estimated by amino acid analysis.

Heparin binding analysis

rTN, rTRIP-A and rM1TN bound to heparin-Sepharose and could be gradient-eluted, producing peaks with absorbance maxima (V_e) at 308, 310 and 345 mM NaCl respectively, whereas rTN23 and rTN3 did not bind in 100 mM NaCl (Figure 2 and Table 1). Neither Ca^{2+} nor EDTA affected the elution profiles with respect to peak shape or V_e (results not shown). Each rTN derivative was eluted quantitatively from the heparin-Sepharose column in the sense that no protein was detectable in the washing step between column loadings. nTN eluted at 223 mM NaCl as a broader peak. Carbohydrate charge shielding of lysine residues probably accounted for the weaker binding of native TN to heparin; the known heterogeneity in the glycosyl prosthetic group in nTN is likely to have accounted for peak broadening.

Each replacement of single lysine residues encoded in exon 1 with alanine resulted in a decreased affinity for heparin (Table 1). However, the change in affinity was apparently influenced by the sequence position of the lysine residue in the structure, in addition to the general effect of removing a single positive charge. The effect of replacing Lys-9 by Ala (V_e at 205 mM NaCl) was more pronounced than that of replacing Lys-6 or Lys-8 (V_e at 232 and 228 mM NaCl respectively). Replacement of Pro-7 with Ala had no effect on affinity for heparin (V_e at 313 mM NaCl).

The rM1TN chimaeric construct essentially represents a replacement of the hydrophobic residues in the human exon 1 sequence with alanine (Figure 3). Notably, the chimaeric construct bound more strongly than wild-type human rTN to heparin-Sepharose.

Table 1 Binding of rTN derivatives and nTN to heparin-Sepharose

The elution volume, V_e, and the full width at half maximum (FWHM) were determined as described in the Experimental section. V_e and FWHM are shown translated from volume units into gradient position (mM NaCl) in accordance with a conductivity standard curve.

TN derivative	V_e (mM NaCl)	FWHM (mM NaCl)
rTN	308	41
nTN	223	76
rTRIP-A	310	29
rTN23	100	9
rTN3	100	7
rM1TN	345	35
rTN K6A	232	28
rTN P7A	313	32
rTN K8A	228	30
rTN K9A	205	33
rTN K14A	219	26
rTN K15A	213	30
rTN K21A	273	28
rTN K27A	289	35
rTN K41A	308	35

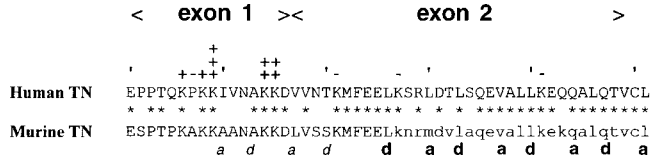

Figure 3 Functional and structural features of the TN N-terminal region

Alignment of the N-terminal regions (residues 1–51) of human and murine TN. Asterisks denote identical residues. Exon boundaries and the coiled-coil heptad repeats (**a**, **d**) are indicated, as well as the putative heptad repeat (*a*, *d*). Residues 1–26 of the murine TN sequence, which replaced the corresponding human sequence in the chimaeric construct rM1TN, are shown in capital letters. The importance to heparin affinity of single-residue mutations is indicated above each residue: + + +, more pronounced; + +, intermediate; +, less pronounced; −, no effect on affinity.

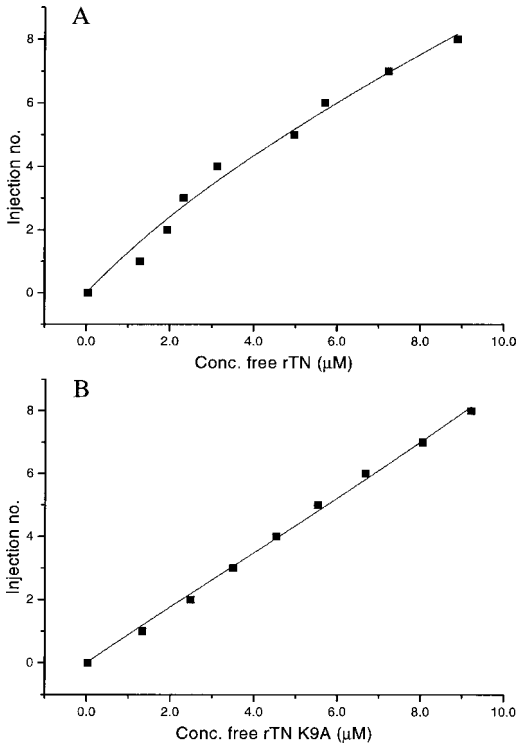

Figure 4 Semi-quantitative affinity binding analysis

Representative titration plots from an experimental series of semi-quantitative heparin-Sepharose affinity analysis. The concentration of measured free ligand is plotted for each addition of ligand in a particular series, as described in the Experimental section. (**A**) Heparin-Sepharose titrated with rTN; (**B**) heparin-Sepharose titrated with rTN K9A.

Replacement of each of the exon-2-encoded lysine residues with alanine exhibited only marginal or no influence on heparin binding affinity. Essentially identical results were obtained for the binding of TN derivatives to immobilized fucoidan (R. H. Lorentsen and J. H. Graversen, unpublished work).

The apparent binding constants for the interaction between heparin-Sepharose and rTN and rTN K9A were determined by using semi-quantitative affinity chromatography as described in the Experimental section. Representative results from the semi-quantitative binding analysis are presented in Figure 4. Three series of experiments were performed with rTN and two with rTN K9A. The apparent K_a' for the binding of rTN was determined as $(1.2 \pm 0.2) \times 10^5\ M^{-1}$ and for the binding of rTN K9A as $(0.5 \pm 0.1) \times 10^5\ M^{-1}$. All fits had χ^2 values in the range of 0.125–0.107. The binding capacity of the heparin-Sepharose was determined as $65 \pm 2.0\ \mu M$ and $43 \pm 11\ \mu M$ respectively.

This study shows that the heparin-binding site is located in a region comprising the N-terminal 16 residues of TN. Notably, our results show that the CRD of TN is not involved in heparin binding. This is demonstrated by the fact that neither rTN3 nor rTN23 was found to bind heparin and that the interaction strength for TN was not affected by Ca^{2+} ions or EDTA. rTN23 is a trimer [1] and it would therefore be expected that a weak affinity site in the CRD, which might have been masked by a stronger interaction with the N-terminal binding site, should become apparent, because trimerization would generate an additive effect by presenting a high local concentration of such binding sites at the heparin-Sepharose surface.

Mutational analysis of the region revealed that all five lysine residues encoded in exon 1 contribute to heparin binding, with Lys-9 showing a stronger and Lys-6 and Lys-8 a weaker contribution to the affinity, and that the lysine residues encoded in exon 2 provided marginal or no contribution to binding affinity. Replacement of the exon-1-encoded hydrophobic residues with alanine seemed not to decrease the binding of heparin.

The crystallographic analysis of TN [15] showed no electron density corresponding to the 26 N-terminal residues, probably owing to accidental proteolytic cleavage during crystallization. The crystal structure does, however, show that residues 26–52 form an α-helical coiled-coil trimerization module. The coiled-coil structure is based on the presence of heptad repeats, mostly with hydrophobic residues in positions *a* and *d* in the sequence (Figure 3). That heptad repeat pattern breaks down between exons 1 and 2 but an additional heptad repeat pattern can tentatively be identified in the N-terminal sequence. The two repeat patterns are out of register by one amino acid residue (close to Phe-23), a perturbation that would be expected to generate a structural discontinuity at the junction of a combined coiled-coil structure [31].

In comparison with other heparin-binding proteins, TN exhibits a relatively low affinity for heparin-Sepharose. The heparin-binding site in TN is devoid of arginine residues; lysine has been shown to contribute less strongly to heparin affinity than arginine [32].

In conclusion, TN interacts with heparin via its N-terminal lysine-rich region. TN, which is encoded in three exons, harbours three main structural and functional features, which reside in separate exons. Exon 1 contains the heparin-binding site, exon 2 is necessary and sufficient for trimerization, and exon 3 encodes the CRD, which is necessary and sufficient for the lysine- and Ca^{2+}-sensitive interaction with kringle 4 in Plg.

We thank Torben Ellebæk Petersen (Department of Molecular and Structural Biology, University of Aarhus, Aarhus, Denmark) for the gift of murine TN cDNA, and Linda Agerholm for skilful technical assistance. The supply of outdated human plasma from the Skejby Hospital Blood Bank (project no. 41) is acknowledged.

REFERENCES

1 Holtet, T. L., Graversen, J. H., Clemmensen, I., Thøgersen, H. C. and Etzerodt, M. (1997) Protein Sci. **6**, 1511–1515
2 Clemmensen, I., Petersen, L. C. and Kluft, C. (1986) Eur. J. Biochem. **156**, 327–333
3 Wewer, U. M., Iba, K., Durkin, M. E., Nielsen, F. C., Loechel, F., Gilpin, B. J., Kuang, W., Engvall, E. and Albrechtsen, R. (1998) Dev. Biol. **200**, 247–259
4 Wewer, U. M., Ibaraki, K., Schjørring, P., Durkin, M. E., Young, M. F. and Albrechtsen, R. (1994) J. Cell Biol. **127**, 1767–1775
5 Iba, K., Sawada, N., Chiba, H., Wewer, U. M., Ishii, S. and Mori, M. (1995) FEBS Lett. **373**, 1–4
6 Christensen, L. and Clemmensen, I. (1989) Histochemistry **92**, 29–35
7 Christensen, L. and Clemmensen, I. (1991) Histochemistry **95**, 427–433
8 Wewer, U. M. and Albrechtsen, R. (1992) Lab. Invest. **67**, 253–262
9 De Vries, T. J., De Wit, P. E., Clemmensen, I., Verspaget, H. W., Weidle, U. H., Bröcker, E. B., Ruiter, D. J. and Van Muijen, G. N. P. (1996) J. Pathol. **179**, 260–265
10 Kluft, C., Jie, A. F. H., Los, P., de Wit, E. and Havekes, L. (1989) Biochem. Biophys. Res. Commun. **161**, 427–433
11 Kluft, C., Los, P. and Clemmensen, I. (1989) Thromb. Res. **55**, 233–238
12 Berglund, L. and Petersen, T. E. (1992) FEBS Lett. **309**, 15–19
13 Graversen, J. H., Lorentsen, R. H., Jacobsen, C., Moestrup, S. K., Sigurskjold, B. W., Thøgersen, H. C. and Etzerodt, M. (1998) J. Biol. Chem. **273**, 29241–29246
14 Drickamer, K. and Taylor, M. E. (1993) Annu. Rev. Cell Biol. **9**, 237–264
15 Nielsen, B. B., Kastrup, J. S., Rasmussen, H., Holtet, T. L., Graversen, J. H., Etzerodt, M., Thøgersen, H. C. and Larsen, I. K. (1997) FEBS Lett. **412**, 388–396
16 Kastrup, J. S., Nielsen, B. B., Rasmussen, H., Holtet, T. L., Graversen, J. H., Etzerodt, M., Thøgersen, H. C. and Larsen, I. K. (1998) Acta Crystallogr. D **54**, 757–766

17 Clemmensen, I. (1989) Scand. J. Clin. Lab. Invest. **49**, 719–725
18 Fuhlendorff, J., Clemmensen, I. and Magnusson, S. (1987) Biochemistry **26**, 6757–6764
19 Jaquinod, M., Holtet, T. L., Etzerodt, M., Clemmensen, I., Thøgersen, H. C. and Roepstorff, P. (2000) Biol. Chem. **380**, 1307–1314
20 Jackson, R. L., Busch, S. J. and Cardin, A. D. (1991) Physiol. Rev. **71**, 481–539
21 Margalit, H., Fischer, N. and Ben-Sasson, S. A. (1993) J. Biol. Chem. **268**, 19228–19231
22 Fromm, J. R., Hileman, R. E., Caldwell, E. E., Weiler, J. M. and Linhardt, R. J. (1997) Arch. Biochem. Biophys. **343**, 92–100
23 Thompson, L. D., Pantoliano, M. W. and Springer, B. A. (1994) Biochemistry **33**, 3831–3840
24 Olson, S. T., Halvorson, H. R. and Björk, I. (1991) J. Biol. Chem. **266**, 6342–6352
25 Olson, S. T. and Björk, I. (1991) J. Biol. Chem. **266**, 6353–6364
26 Faller, B., Mely, Y., Gerard, D. and Bieth, J. G. (1992) Biochemistry **31**, 8285–8290
27 Lookene, A., Chevreuil, O., Østergaard, P. and Olivecrona, G. (1996) Biochemistry **35**, 12155–12163
28 Sørensen, C. B., Berglund, L. and Petersen, T. E. (1995) Gene **152**, 243–245
29 Thøgersen, H. C., Holtet, T. L. and Etzerodt, M. (1994) International Patent WO 94/18227
30 Hogg, P. J. and Winzor, D. J. (1987) Anal. Biochem. **163**, 331–338
31 Lupas, A. (1996) Trends Biochem. Sci. **21**, 375–382
32 Fromm, J. R., Hileman, R. E., Caldwell, E. E., Weiler, J. M. and Linhardt, R. J. (1995) Arch. Biochem. Biophys. **323**, 279–287

Received 3 June 1999/26 November 1999; accepted 11 January 2000