ELSEVIER

FEBS Letters 344 (1994) 242–246

FEBS 13964

FEBS LETTERS

Receptor-binding domain of human α_2-macroglobulin

Expression, folding and biochemical characterization of a high-affinity recombinant derivative

Thor Las Holtet[a], Kåre Lehmann Nielsen[a,b], Michael Etzerodt[a], Søren Kragh Moestrup[c], Jørgen Gliemann[c], Lars Sottrup-Jensen[b], Hans Christian Thøgersen[a,*]

[a]*Laboratory of Gene Expression, Department of Chemistry,* [b]*Department of Molecular Biology, and* [c]*Department of Medical Biochemistry, University of Århus, DK-8000 Århus C, Denmark*

Received 16 February 1994; revised version received 29 March 1994

Abstract

A recombinant version of the receptor binding domain (RBDv) of human α_2-macroglobulin (α_2M) has been expressed in *E. coli* and refolded using a novel iterative procedure. RBDv (Val^{1299}-Ala^{1451}) is extended by 15 residues at the N-terminal side of the Lys^{1313}-Glu papain cleavage site in human α_2M. RBDv contains the intra-chain bridge Cys^{1329}-Cys^{1444} and is soluble and monomeric. Competition experiments with ^{125}I-labelled methylamine-treated α_2M reveal that RBDv binds to the placental receptor for transformed α_2M with a K_d of 8 nM, i.e. the binding affinity of RBDv is of the same order of magnitude as the intrinsic affinity for binding of one domain in transformed α_2M to one receptor molecule.

Key words: α-Macroglobulin; Domain structure; Protein expression; α_2-Macroglobulin receptor

1. Introduction

The α_2-macroglobulins (α_2Ms) constitute a family of large proteinase inhibitors found in the blood of vertebrates and invertebrates, and in bird and reptile egg-white. Most α_2Ms including human α_2M are tetrameric proteins consisting of approx. 1450 residue subunits. The inhibitory function of α_2M is achieved primarily through entrapment of the protease molecule. This is caused by a large-scale structural rearrangement, known as transformation, initiated by proteolytic cleavage within the exposed 'bait regions' of α_2M. The subunits of α-macroglobulins from most species contain an intra-chain β-cysteinyl-γ-glutamyl thiol ester which becomes highly susceptible to nucleophilic attack by e.g. lysyl side chains upon transformation. This often leads to covalent immobilization of the entrapped protease within its molecular cage. For reviews on α_2Ms see [1,2]. Transformation of α_2M, either achieved by proteolytic cleavage of the bait region or by cleavage of the thiol esters with primary aliphatic amines, elicits the exposure of a receptor-binding module, concealed in native α_2M [3–5].

The receptor for transformed α_2M is termed α_2MR/LRP [6–8]. The 515 kDa α-chain of α_2MR/LRP is ligand binding whereas its 85 kDa β-chain is membrane spanning [9–11]. α_2MR/LRP is found in many tissues, including fibroblasts, adipocytes, macrophages and hepatocytes [3,5,12,13].

The receptor-binding domain of α_2M appears to reside in the C-terminal region of each polypeptide chain [14]. This is corroborated by the isolation of a C-terminal fragment obtained by limited proteolysis of α_2M-MA, RBD (residues 1314–1451), which can compete for transformed α_2M binding sites in α_2MR/LRP, albeit with an affinity of only about 1–2% of that exhibited by transformed α_2M [15–17]. The corresponding domain from rat α_1M (residues 1313–1451, human α_2M numbering) has recently been expressed in a bacterial system and shown to retain biological activity of the same magnitude as the proteolytically derived fragments [18].

Here we describe the bacterial expression, folding and biochemical characterization of a 153-residue polypeptide (RBDv, Val^{1299}-Ala^{1451}) representing RBD extended by 15 residues at the N-terminal side of the Lys^{1313}-Glu

*Corresponding author. Laboratory of Gene Expression, Gustav Wieds Vej 10, DK-8000 Århus C, Denmark. Fax: (45) (86) 20 12 22.

Abbreviations: α_2M, α_2-macroglobulin; α_2MR/LRP, receptor for transformed α_2-macroglobulin/low density lipoprotein receptor-related protein; α_2M-MA, methylamine-treated α_2-macroglobulin; RBD, receptor binding domain of α_2-macroglobulin (Glu^{1314}-Ala^{1451}); RBDv, expressed variant of receptor binding domain (Val^{1299}-Ala^{1451}); PCR, polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; NTA, nitrilo triacetic acid.

SSDI 0014-5793(94)00349-Z

cleavage site in human α_2M. We show that in vitro refolding of this polypeptide can efficiently generate a compact, correctly disulfide-bridged molecule that is monomeric in solution and exhibits binding to α_2MR/LRP with an affinity approx. 10 times higher than that previously determined for the RBD fragment.

2. Materials and methods

2.1. Construction of the expression plasmid pT_7H_6ScFX-α_2M-RBDv

A 462 bp DNA fragment encoding residues Val[1299]-Ala[1451] of human α_2M was amplified, essentially according to [19], over 30 cycles using an ABACUS thermocycler (Hybaid Ltd). 1 U *Taq* DNA Polymerase (HT Biotechnology), approx. 50 pg plasmid DNA, containing a full-length α_2M cDNA insert (isolated and kindly provided by Dr. T. Kristensen, Department of Chemistry, University of Århus) as template, and 80 pmole of each of the two primers were used (the underlined nucleotides encode a FX_a cleavage site):

5′-CCTGGATCCATCGAGGGTAGGGTCTACCTCCAGACATCCT-3′ and
5′-CCGAAGCTTCAAGCATTTCCAAGATC 3′.

The amplified DNA fragment was cut with *Bam*HI and *Hin*dIII (Boehringer), isolated after agarose gel eletrophoresis, and ligated into the *E. coli* expression vector pT_7H_6 [20]. After subcloning of the DNA fragment the plasmid was modified by insertion in the *Bam*HI site of an oligonucleotide (Sc) encoding the amino acid sequence GSLPQNPFSTSSTLPRS. The nucleotide sequence of the plasmid insert was verified using the Sequenase ver. 2.0 DNA sequencing kit (USB Corp.).

2.2. Expression in E. coli *of recombinant RBDv protein*

Recombinant RBDv protein was produced by expressing the plasmid pT_7H_6ScFX-α_2MRBDv, which encodes a FX_a cleavable fusion protein [21], in *E. coli* DH1 cells as described [22]. At OD_{600} = 0.8 exponentially growing cultures at 37°C were infected with bacteriophage λCE6 at a multiplicity of approx. 5. 30 min after infection rifampicin (Sigma) was added and the cultures were grown at 37°C for another 3 h before cells were harvested by centrifugation. Cells were lysed by osmotic shock and sonication and total cellular protein extracted into phenol (adjusted to pH 8.0 with Tris base). Protein was precipitated from the phenol phase by addition of 2.5 vols. of ethanol and isolated by centrifugation. The protein pellet was dissolved in 50 mM Tris-HCl, 6 M guanidinium chloride, 100 mM dithioerythriol, pH 8.0. Following buffer exchange into 50 mM Tris-HCl, 8 M urea, 0.5 M NaCl, 10 mM 2-mercaptoethanol, 2 mM methionine, pH 8.0, by gel filtration on Sephadex G-25 (Pharmacia) the crude protein preparation was applied to a 40 ml Ni^{2+} activated NTA-Sepharose (Pharmacia) column [23]. The Ni^{2+} NTA-Sepharose column was then washed with 50 mM Tris-HCl, 6 M guanidinium chloride, 10 mM 2-mercaptoethanol, 2 mM methionine, pH 8.0 until A^{280} of the eluate was stable (approx. 5 column vols.).

2.3. Refolding and processing of the α_2M-RBDv fusion protein

The fusion protein was refolded in vitro, while immobilized on the Ni^{2+} NTA-Sepharose column, using an iterative refolding procedure (TLH, ME, and HCT, Patent application no. DK0130/93, DK0139/93; manuscript in preparation). After completion of the iterative folding procedure the fusion protein was eluted from the Ni^{2+} NTA-Sepharose column with 50 mM Tris-HCl, 0.5 M NaCl, 10 mM EDTA, pH 8.0. Fusion protein that was aggregated and precipitated on the Ni^{2+} NTA-Sepharose column was eluted with 50 mM Tris-HCl, 8 M urea, 0.5 M NaCl, 10 mM 2-mercaptoethanol, 2 mM methionine, pH 8.0.

Recombinant RBDv was liberated from the N-terminal fusion tail by cleavage with 1/50 (w/w) of the restriction protease FX_a at room temperature for 4 h. After cleavage RBDv was isolated from uncleaved fusion protein, liberated fusion tail and FX_a by gel-filtration on Sephadex G-25 in 50 mM Tris-HCl, 10 mM NaCl, pH 8.0, followed by ion-exchange chromatography on Q-Sepharose (Pharmacia). RBDv was eluted with a linear gradient over 10 column vols. from 10 mM to 500 mM NaCl in 10 mM Tris-HCl, pH 8.0. RBDv eluted at 150 mM NaCl.

2.4. Amino acid and N-terminal sequence analysis

After hydrolysis for 20 h at 110°C in vacuo with 6 M HCl, 0.1% phenol, 5% thioglycollic acid the amino acid composition was determined by cation-exchange chromatography [24] modified to permit determination of half-cystine as cysteine [25].

N-Terminal sequence analysis was done by Edman degradation in an Applied Biosystems 477A instrument equipped with a 120A on-line chromatograph using a polybrene-coated glass filter and Normal-1 cycles.

2.5. Binding of α_2M-MA and RBDv to purified human α_2MR/LRP

MaxiSorp microtiter wells (Nunc) were coated with α_2MR/LRP by incubation for 2 h at room temperature with 100 μl receptor (approx. 0.5 μg/ml) in 50 mM $NaHCO_3$, pH 9.6. After blocking with 5% bovine serum albumin for 2 h, the plates were washed 3 times with 10 mM sodium phosphate, 140 mM NaCl, pH 7.4, containing 0.05% Tween-20. Incubations with [^{125}I]α_2M-MA [10,26] and different concentrations of unlabelled α_2M-MA or RBDv were performed for 16 h at 4°C in 100 μl 10 mM HEPES, 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2% bovine serum albumin, pH 7.4. Following washing, bound radioactivity was released by the addition of 2 × 150 μl 10% SDS and determined. Non-specific binding of [^{125}I]α_2M-MA to wells not coated with α_2MR/LRP was likewise determined and subtracted from the values determined in the binding experiments. Non-specific binding was less than 0.5% of binding of the added tracer.

3. Results

3.1. Rationale for studying RBDv

It was found previously [16] that by exposing RBD isolated by proteolytic digestion of α_2M-MA to 6 M guanidinium chloride followed by removal of the denaturant by dialysis the binding affinity for α_2MR/LRP could not be fully restored. In addition (unpublished) it was found that RBD freeze-dried from NH4HCO3 was poorly soluble in non-denaturing buffers at pH 7–8; the material that did go into solution was strongly aggregated, as determined from gel filtration.

These results, in conjunction with the ^{1}H NMR and CD spectra of RBD (N.K. Thomsen, LS-J and F.M. Poulsen, unpublished), indicated that RBD might be a marginally stable domain. Indeed, refolding of recombinant RBD failed to produce a monomeric product (not shown).

In the α_2M family the sequence immediately upstream of the Lys[1313]-Glu cleavage site is strongly conserved and contains a fair number of hydrophobic residues [1,2]. Therefore, this stretch could conceivably be an integral part of the receptor-binding domain and might therefore be required to establish and maintain its correctly folded structure. Since α_2M contains a paired Cys residue at position 1298 it was decided to investigate the properties of expressed polypeptide starting at Val[1299] (RBDv).

3.2. Construction of the expression plasmid pT_7H_6ScFX-α_2MRBDv

The 462 bp reading frame encoding the 153 residue RBDv was, due to the design of the 5′ PCR-oligonucleotide primer, linked at the 5′ end to a nucleotide sequence encoding a cleavage site (GSIEGR) for FX_a [21]. The amplified DNA fragment was subcloned into the *E. coli*

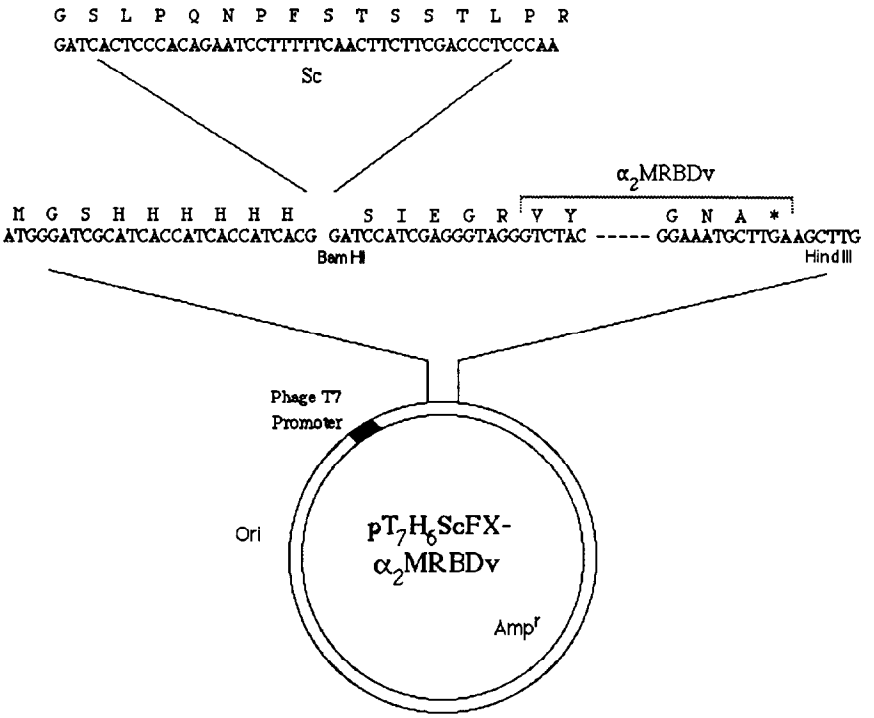

Fig. 1. Map of the *E. coli* expression vector pT_7H_6ScFX-α_2MRBDv. After subcloning of the RBDv PCR-fragment into the *Bam*HI and *Hin*dIII restriction sites of pT_7H_6 [20] the oligonucleotide (Sc) was ligated into the *Bam*HI site. The Sc nucleotide sequence encodes an amino acid sequence capable of accelerating FX_a cleavage (unpublished).

expression vector pT_7H_6 [20]. The N-terminal fusion tail of the expression plasmid was further modified in the *Bam*HI restriction site by insertion of an oligonucleotide encoding the peptide sequence GSLPQNPFSTSSTLPR (Sc). Insertion of that peptide N-terminal of the FX_a cleavage site enhances the rate of FX_a cleavage required to liberate the recombinant protein (unpublished). A map of the expression plasmid pT_7H_6ScFX-α_2MRBDv is shown in Fig. 1.

3.3. Expression, refolding, and processing of the RBD fusion protein

The expression in *E. coli*, purification, in vitro refolding, and processing of the RBDv fusion protein was analysed by SDS-PAGE as shown in Fig. 2. Approx. 50% of the fusion protein material was eluted from the Ni^{2+} NTA-Sepharose column with the non-denaturing elution buffer (Fig. 2, lane 2). Ninety per cent of the soluble fusion protein material appeared monomeric yielding an overall efficiency of the folding procedure of approx. 45%. The yield of fully processed and purified RBDv protein was 0.5 mg/l of culture.

3.4. Characterization of RBDv

The amino acid composition of RBDv was determined after acid hydrolysis in the presence of thioglycollic acid is shown in Table 1. Apart from Asx and Gly a good agreement with the composition expected for Val^{1299}-Ala^{1451} is evident. Further, N-terminal sequence analysis (23 residues determined) revealed a perfect match with residues Val^{1299}-Gly^{1321} (not shown).

From reducing SDS-PAGE (Fig. 2, lane 5) the size of RBDv was estimated at 17 kDa (expected 17.1 kDa). As further seen from Fig. 2, lane 4 RBDv was monomeric in non-reducing conditions, and its increased mobility compared with the reduced state showed that the Cys^{1329}-

Table 1
Amino acid composition of RBDv[a]

Amino acid	Found	Expected
Asx	16.20	15
Thr	9.51	10
Ser	15.46	16
Glx	15.65	15
Pro	9.24	9
Cys	2.08	2
Gly	5.79	4
Ala	9.31	9
Val	15.28	16
Met	2.57	3
Ile	6.24	7
Leu	15.00	15
Tyr	7.69	8
Phe	6.99	7
His	3.15	3
Lys	9.89	10
Arg	4.01	4
Trp	0.00	0

[a] Molar rations assuming 15 Leu residues, average of four determinations.

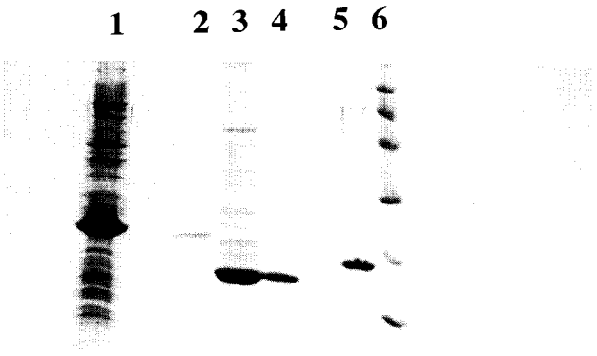

Fig. 2. SDS-PAGE analysis (18% gel) of steps in the purification of RBDv. Lane 1, crude cell extract; lane 2, eluate from Ni^{2+} column (fusion protein); lane 3, RBDv concentrated by ultrafiltration after cleavage with FX_a; lanes 4 and 5, RBDv purified by ion exchange chromatography; lane 6, size markers (94, 67, 43, 30, 20, and 14 kDa from top to bottom). lanes 2–4 were non-reduced, lanes 1, 5 and 6 were reduced.

Cys^{1444} intra-chain bridge had been formed during refolding. Upon gel filtration on a calibrated column of Superdex 75 in non-denaturing conditions RBDv eluted as a monomeric protein of approx. 18 kDa. Hence, from the criteria employed, RBDv is likely to constitute a domain with a well-defined tertiary structure containing the bridge Cys^{1329}-Cys^{1444}.

3.5. Analysis of receptor binding

Fig. 3 shows the concentration dependence of inhibition of binding of 10 pM [^{125}I]α_2M-MA to α_2MR/LRP by unlabelled α_2M-MA and RBDv. Half-maximal binding was seen at about 100 pM α_2M-MA and at 8 nM RBDv. In contrast, for RBD isolated from α_2M-MA by proteolytic digestion, half-maximal binding occurred at approx. 100 nM in a similar experiment [10] (Fig. 3). The inhibition curves for RBDv and RBD were fitted according to a simple one-receptor model with apparent K_d values of 8 and 99 nM, respectively, whereas the curve for the tetravalent α_2M-MA was fitted in accordance with its high and low affinity modes [10]. The high affinity mode (K_d approx. 40 pM) has been explained by the interaction of tetravalent α_2M-MA with two or more receptors, whereas the low affinity mode (K_d approx. 2 nM) is thought to represent the intrinsic affinity for binding of one domain in α_2M-MA to one receptor molecule.

4. Discussion

The presence in α_2Ms of a module which becomes available for receptor recognition upon transformation is an element in a highly sophisticated mechanism for scavenging many foreign or spent proteases. The molecular events resulting in recognition of transformed α_2M by α_2MR/LRP and the subsequent endocytosis and degradation of α_2M–protease complexes are not well understood.

The receptor binding domain (RBD, Glu^{1314}-Ala^{1451}) of α_2M has previously been available only in the form of a fragment obtained by proteolysis of α_2M-MA [15–17]. To permit studies of the recognition of transformed α_2M by α_2MR/LRP by amino acid replacement strategies we have expressed a variant of RBD (RBDv, Val^{1299}-Ala^{1451}) which contains 15 additional residues (Val^{1299}-Lys^{1313}). RBDv was produced in *E. coli* and in vitro refolded using a novel iterative refolding procedure.

By the criteria employed (SDS-PAGE in the absence and presence of reductant, gel chromatography, compositional and partial sequence analysis) RBDv was judged to constitute correctly folded material.

When assayed for in vitro binding to α_2MR/LRP RBDv was found to specifically displace 125I-α_2M-MA, corresponding to an apparent K_d of 8 nM. It was determined previously, using RBD obtained by proteolysis of tetrameric α_2M-MA from different species, that K_d was approx. 100–300 nM [10,15–17]. The only exception was RBD from the monomeric rat α_1-inhibitor-3 (K_d = 10 nM) [17]. Hence, recombinant RBDv represents a domain which binds to α_2MR/LRP with an affinity at least 10-fold higher than RBD previously isolated from tetrameric α_2Ms, and of the same order of magnitude as that of a single domain in α_2M-MA.

The expression in *E. coli* of a recombinant homologue of RBD derived from rat α_1M was recently described [18].

Based on $E_{1\%,280\ nm,1\ cm}$ = 11.5 (which may represent a

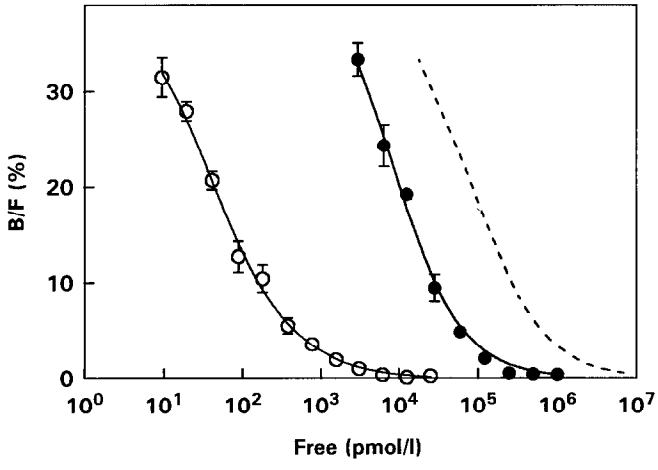

Fig. 3. Competition of RBDv (●) and α_2M-MA (○) with 10 pM [^{125}I]α_2M-MA for binding to purified placental α_2MR/LRP immobilised to microtiter wells. Binding was measured after incubation for 16 h at 4°C. The dashed line shows competition of RBD with [^{125}I]α_2M-MA for α_2MR/LRP in a similar experiment [10].

near 2-fold overestimation [16,27]) K_d was determined to be approx. 20 nM for this preparation.

In the protocol used for expressing RBD from α_1M the protein was located intracellularly and was soluble [18]. Probably due to the absence of the bridge Cys^{1329}-Cys^{1444} in α_1M (both residues being replaced by Phe residues) the recombinant α_1M RBD folded spontaneously.

The additional 15 residues in the present RBDv construct appear not only to permit refolding under the conditions employed, so that the intra-chain Cys^{1329}-Cys^{1444} bridge forms, but they also subtly influence the conformation of the folded protein as reflected in an increased affinity of RBDv for α_2MR/LRP.

The present expression and refolding procedure will form the basis for introducing specific mutations aimed at defining the determinants for receptor recognition.

Acknowledgements: This work was supported by grants from the Danish Biotechnology Programme (Protein Engineering and Biomembrane Research Centres).

References

[1] Sottrup-Jensen, L. (1987) The Plasma Proteins (Putnam, F.W., ed.) 2nd ed., vol. 5, pp. 191–291
[2] Sottrup-Jensen, L. (1989) J. Biol. Chem. 264, 11539–11542
[3] Van Leuven, F., Cassiman, J.-J. and Van Den Berghe, H. (1981) J. Biol. Chem. 256, 9016–9022
[4] Imber, M.J. and Pizzo, S.V. (1981) J. Biol. Chem. 256, 8134–8139
[5] Gliemann, J., Larsen, T.R. and Sottrup-Jensen, L. (1983) Biochim. Biophys. Acta 756, 230–237
[6] Kristensen, T., Moestrup, S.K., Gliemann, J., Bendtsen, L., Sand, O. and Sottrup-Jensen, L. (1990) FEBS Lett. 276, 151–155
[7] Strickland, D.K., Ashcom, J.D., Williams, S., Burgess, W.H., Migliorini, M. and Argraves, W.S. (1990) J. Biol. Chem. 265, 17401–17404
[8] Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gausepohl, H. and Stanley, K.K. (1988) EMBO J. 7, 4119–4127
[9] Herz, J., Kowal, R.C., Goldstein, J.L. and Brown, M.S. (1990) EMBO J. 9, 1769–1776
[10] Moestrup, S.K. and Gliemann, J. (1991) J. Biol. Chem. 266, 14011–14017
[11] Herz, J., Goldstein, J.L., Strickland, D.K., Ho, Y.K. and Brown, M.S. (1991) J. Biol. Chem. 266, 21232–21238
[12] Kaplan, J., Ray, F.H. and Keogh, E. (1981) J. Biol. Chem. 256, 7705–7707
[13] Moestrup, S.K., Gliemann, J. and Pallesen, G. (1992) Cell Tissue Res. 269, 375–382
[14] Van Leuven, F., Marynen, P., Cassiman, J.-J. and Van Den Berghe, H. (1986) J. Biol. Chem. 261, 6933–6937
[15] Van Leuven, F., Marynen, P., Sottrup-Jensen, L., Cassiman, J.-J. and Van Den Berghe, H. (1986) J. Biol. Chem. 261, 11369–11373
[16] Sottrup-Jensen, L., Gliemann, J. and Van Leuven, F. (1986) FEBS Lett. 205, 20–24
[17] Enghild, J.J., Thøgersen, I.B., Roche, P.A. and Pizzo, S.V. (1989) Biochemistry 28, 1406–1412
[18] Salvesen, G., Quan, L.T., Enghild, J.J., Snipas, S., Fey, G.H. and Pizzo, S.V. (1992) FEBS Lett. 313, 198–202
[19] Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) Science 239, 487–491
[20] Christensen, J.H., Hansen, P.K., Lillelund, O. and Thøgersen, H.C. (1991) FEBS Lett. 295, 181–184
[21] Nagai, K. and Thøgersen, H.C. (1987) Methods Enzymol. 152, 461–481
[22] Studier, F.W. and Moffat, B.A. (1986) J. Mol. Biol. 189, 113–130
[23] Hochuli, E., Bannwarth, W., Döbeli, H., Gentz, R. and Stüber, D. (1988) Bio/Technology 6, 1321–1325.
[24] Barkholt, V. and Jensen, A.L. (1989) Anal. Biochem. 177, 318–322
[25] Sottrup-Jensen, L. (1993) Biochem. Mol. Biol. Int. 30, 789–794
[26] Nykjær, A., Petersen, C.M., Møller, B., Jensen, P.H., Moestrup, S.K., Holtet, T.L., Etzerodt, M., Thøgersen, H.C., Munch, M., Andreasen, P.A. and Gliemann, J. (1992) J. Biol. Chem. 267, 14543–14546
[27] Gill, S.C. and Von Hippel, P.H. (1989) Anal. Biochem. 182, 319–326