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Ligand interaction of human α2-macroglobulin–α2-macroglobulin receptor studied by partitioning in aqueous two-phase systems[1]

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Abstract

Alpha 2-macroglobulin (α2-M) is a major proteinase inhibitor in human blood and tissue. Besides its antiproteolytic potential, α2-M was found to modulate antigen- and mitogen-driven immune responses and cell growth by binding and transporting distinct cytokines, growth factors and hormones. The inhibitor is cleared from circulation by binding to a multifunctional cellular receptor present on different cell types. α2-M, as well as its receptor, are capable of binding a variety of ligands. In the present study we have applied aqueous two-phase systems to analyze the interaction of IL-1β and α2-M receptor to different forms of α2-M. The partition of IL-1β was changed by addition of transformed α2-M to the two-phase systems rather than by the native inhibitor. The interaction between IL-1β and α2-M was enhanced by divalent cations. In addition, the complex formation between ^{125}I-labelled receptor and α2-M could clearly be demonstrated by partitioning. In the presence of divalent cations, transformed α2-M, in contrast to the native inhibitor, effectively changed the partition of the receptor. However, the observed alteration of the partition coefficient was found to be less compared with the values obtained by partitioning of the receptor in the presence of whole plasma containing the inhibitor in equivalent concentrations. The results indicate that other components of the plasma exist which competitively bind to the receptor but independent of Ca^{2+}-ions.

Keywords: Aqueous two-phase system; Partitioning; Ligand interaction; α_2-Macroglobulin; Proteinase

1. Introduction

Alpha 2-macroglobulin (α2-M) is a major proteinase inhibitor in human blood and tissue. Upon the attack of different proteinases rising from the blood clotting cascade, or cell destruction of bacterial origin, the native inhibitor becomes transformed giving rise to structural and conformational rearrangements and to the exposition of previously hidden receptor-recognition sites [1,2]. The transformed inhibitor is then rapidly endocytosed by a membrane-bound receptor present on different cells e.g. macrophages, fibroblasts, neuronal cells, hepatocytes [3,4].

Besides its antiproteolytic potential, α2-M was found to modulate mitogen- and antigen-driven immune responses and cell growth, probably by binding and transporting distinct cytokines, growth factors and hormones [5–7]. Binding to α2-M is thought to confer protection to proteinases, prolongation of circulation time or transport into different cells.

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[1]Dedicated to Prof. G. Kopperschläger (Leipzig) on the occasion of his 60th birthday.

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The receptor for α2-M (α2-M-R) has recently been cloned as a large membrane-associated protein consisting of a large extracellular domain (515 kDa) and a non-covalently attached membrane-spanning domain (85 kDa) [8]. A receptor-associated 39 kDa protein is known to regulate the binding activity of the receptor [9]. Recently, the α2-M-R has been demonstrated to be identical to the low density lipoprotein receptor-related protein (LRP) [10]. This receptor is involved in binding of chylomicron remnants, lipoprotein lipase and lactoferrin [11,12]. In addition, this multifunctional receptor was also found to bind and internalize pro-urokinases–inhibitor complexes and tissue plasminogen activator–inhibitor complexes [13,14].

The interaction of α2-M and α2-M-R with ligands has preferentially been studied in solid-phase experiments by ligand blotting. However, adsorption to solid phases often changes the structure of immobilized proteins. Previous studies revealed that adsorption of α2-M to blotting membranes causes transformation of the native molecule [15]. This prompted us to study the ligand interaction of α2-M and α2-M-R in free solution by partitioning in aqueous two-phase systems. Such systems are composed of poly(ethylene glycol) (PEG) and dextran (Dx) and are known to be very biocompatible to cells and proteins [16–18]. In this study we have analyzed the interaction of IL-1β and purified α2-M-R to different forms of α2-M. The results obtained by partitioning were compared with some data obtained by solid-phase assays.

2. Experimental

2.1. Purification of α2-macroglobulin

Native α2-M was purified to homogeneity by Zn^{2+}-chelate chromatography and gel filtration [15]. The α2-M-R was isolated from human placenta by affinity chromatography as described recently [19].

2.2. Transformation of α2-macroglobulin

Native α2-M or fresh human serum were transformed by treatment with 0.2 *M* methylamine (MA) (Sigma, St. Louis, MO, USA) in 0.1 *M* phosphate buffer, pH 8.0, for 2 h at room temperature. This treatment causes a conformational change in the inhibitor molecule leading to interruption of internal thiolesters, the liberation of free SH-groups and the exposition of receptor recognition sites on the surface [2]. Excess of MA was removed by extensive dialysis against 10 m*M* sodium phosphate buffer. The concentration of native and transformed α2-M in samples was determined by ELISA as previously described [15]. Nascent SH-groups of α2-M emerging by methylamine treatment were blocked by reacting with 10 m*M* iodoacetamide for 30 min followed by dialysis against 10 m*M* sodium phosphate buffer.

2.3. Radiolabelling

The purified receptor was radiolabelled with 0.5 mCi ^{125}I (Amersham, Darmstadt, Germany) according to the chloramine-T method as described by Hunter and Greenwood [20]. Radiolabelled ^{125}I-IL-1β was purchased from Amersham.

2.4. Aqueous two-phase partition

Aqueous two-phase systems (2 g) were prepared from stock solutions of PEG 6000 (Serva, Heidelberg, Germany), Dextran T 70 (Pharmacia, Uppsala, Sweden) and buffer, respectively, to obtain the desired concentrations as given in the legends of the figures. Prior to partitioning the interacting proteins were pre-incubated at 37°C for certain times as given in the legends of the figures. After mixing the two-phase systems phase separation was achieved by centrifugation at 2000 *g*. Volumes of 500 μl were withdrawn from the top and bottom phases, respectively, and were analyzed for protein content or radioactivity. Each partition experiment was performed in triplicate.

The partition coefficient, *K*, is defined as the ratio of radioactivity or protein content in the top and the bottom phase, respectively.

2.5. Solid phase partition

Titer plates (Nunc, Wiesbaden-Biebrich, Germany) were coated with purified α2-M-R (2 μg/ml) in coating buffer (20 m*M* Hepes, 0.15 *M* NaCl, 5

m*M* $CaCl_2$, 1 m*M* $MgCl_2$, pH 7.4) at 4°C overnight. After washing (three times) the plates with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) the samples were incubated at 37°C for 1 h. After repeated washing the bound α2-M was detected using POD-conjugated anti-α2-M from rabbit and *o*-phenylenediamine/H_2O_2 as substrates.

3. Results and discussion

3.1. Ligand binding to α2-macroglobulin

Due to its properties to bind different cytokines, growth factors and hormones α2-M was assumed to play an important role in regulation of the cell growth and cell differentiation [21]. Two different forms of α2-M are present in plasma or tissue which have different structural and functional properties. Native α2-M is the dominating form which, upon proteolytic attack, undergoes a conformational transition leading to the interruption of internal thiolesters and expression of receptor-recognition sites [2–4], as well as expression of hydrophobic pockets on the protein surface [22]. Similar conformational changes can be achieved by treatment with primary amines such as methylamine. Native and methylamine-treated α2-M (α2-M-MA) differ with respect to their binding properties towards cytokines and growth factors. However, due to the very labile structure of native α2-M no complete discrimination could be obtained in many instances.

Fig. 1 demonstrates the feasibility of aqueous two-phase systems for studying binding of a cytokine, e.g. IL-1β, to α2-M under very mild conditions. The two-phase systems used were composed of 5% (w/w) PEG 6000, 7.5% (w/w) Dx T70 and buffer. The *K*-values of radiolabelled IL-1β, α2-M and α2-M-MA in the absence of the interacting species were 0.92, 0.16 and 0.17, respectively. Upon addition of increasing concentrations of transformed α2-M (α2-M-MA) the *K*-value of IL-1β was significantly diminished due to complex formation. According to the large difference in molecular mass (IL-1β, 17 kDa; α2-M, 720 kDa) the partition of the complex is assumed to be mainly governed by the surface properties of α2-M-MA, thus the *K*-value is expected to approach to the *K*-value of α2-M-MA. In order to test whether the cytokine is bound to the

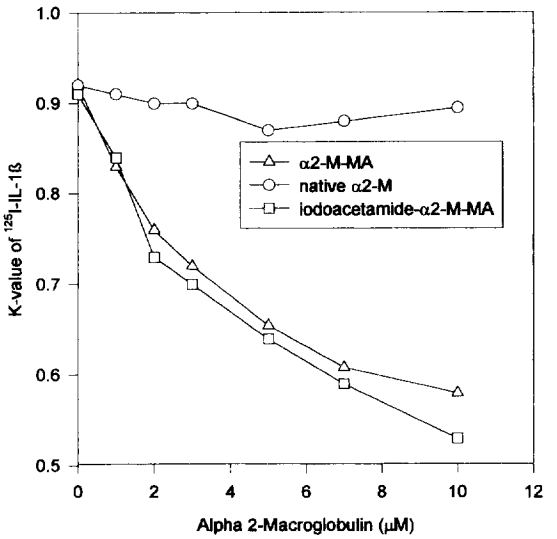

Fig. 1. Effect of α2-M on partition of IL-1β: 41 p*M* ^{125}I-labelled IL-1β were partitioned in two-phase systems (2 g) composed of 5% PEG 6000, 7.5% Dx T70, 0.15 *M* NaCl, 10 m*M* sodium phosphate buffer, pH 7.4, in dependence on increasing concentrations of α2-M (○), α2-M-MA (△), and iodoacetamide-treated α2-M-MA (□). The radiolabelled IL-1β and the different forms of α2-M, respectively, were pre-incubated at 37°C for 3 h before adding to the equilibrated phase system.

liberated SH-groups of thiolesters, these groups were blocked by treatment of α2-M-MA with iodoacetamide. As seen, this modification had no major influence on partition of IL-1β when compared with α2-M-MA. Thus, binding of IL-1β to α2-M-MA most probably involves sites other than the thiolester sites of the inhibitor. No binding of IL-1β to native α2-M could be observed.

α2-M is known to bind and transport Zn^{2+} ions in plasma [23]. Whether divalent cations may influence the binding of IL-1β to α2-M-MA was addressed in Fig. 2. Clearly, in the presence of Zn^{2+} the complex formation between α2-M-MA and IL-1β was strongly increased as indicated by the lower *K*-value of the IL-1β compared with systems without Zn^{2+}. Surprisingly, even Ca^{2+} favoured the binding of IL-1β to α2-M-MA. It was found that EDTA, when added at the end of the incubation of IL-1β, α2-M-MA and Zn^{2+}, could only partially reverse the Zn^{2+}-mediated complex formation (Fig. 3). However, when the interacting proteins were incubated together with EDTA, the Zn^{2+}-effect was abolished. These results indicate that different binding modes of

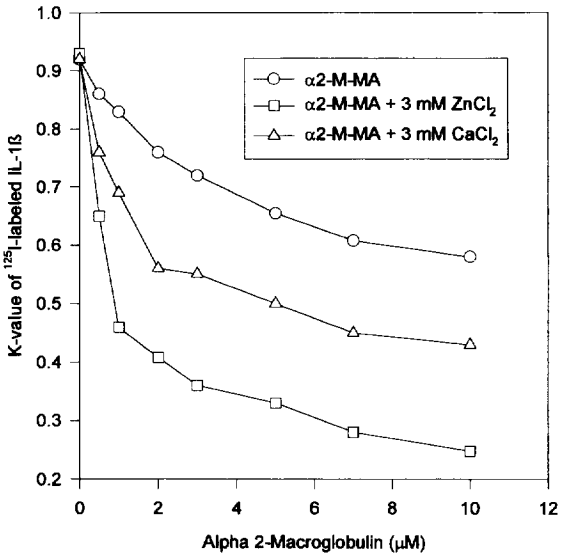

Fig. 2. Effect of divalent cations on binding of IL-1β to α2-M-MA: 41 pM ^{125}I-labelled IL-1β were partitioned in two-phase systems (2 g) composed of 5% PEG 6000, 7.5% Dx T70, 0.15 M NaCl, 10 mM sodium phosphate buffer, pH 7.4, in the absence (○) and the presence of 3 mM $ZnCl_2$ (□) or $CaCl_2$ (△) and increasing concentrations of α2-M-MA. The radiolabelled IL-1β, respective amounts of α2-M-MA and divalent cations were pre-incubated at 37°C for 3 h before adding to the phase system.

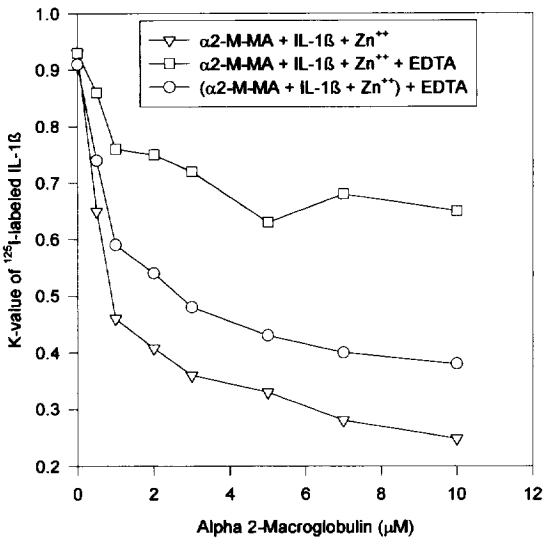

Fig. 3. Effect of EDTA on Zn^{2+}-mediated binding of IL-1β to α2-M-MA: 41 pM ^{125}I-labelled IL-1β were partitioned in two-phase systems (2 g) composed of 5% PEG 6000, 7.5% Dx T70, 0.15 M NaCl, 3 mM $ZnCl_2$, 10 mM sodium phosphate buffer, pH 7.4, and increasing concentrations of α2-M-MA. Mixtures of ^{125}I-IL-1β, α2-M-MA and Zn^{2+} were pre-incubated at 37°C for 3 h in the absence of 3 mM EDTA (△), in the presence of 3 mM EDTA (□), or 3 mM EDTA was added at the end of the incubation time (○).

IL-1β to α2-M-MA may exist. Complementary experiments revealed that Zn^{2+}-ions also mediate binding of IL-1β to other proteins such as human serum albumin (not shown here). However, this interaction could completely be reversed by EDTA and is assumed to be unspecific. Borth and Luger [6] described binding of IL-1β to α2-M through a thiol–disulfide exchange reaction. However, we could not corroborate these results. Currently, the biological role of Zn^{2+}-ions for interaction of IL-1β and transformed α2-M is obviously not clear and needs further study. As known, Zn^{2+}-ions can mediate protein or peptide interactions by forming coordinate bondings or bridges between His- or SH-residues [24]. Such chelating effects of transition metal ions have also been exploited for protein separation by immobilized metal-ion affinity partitioning [25].

3.2. Ligand binding to the α2-M receptor

Strong sequence homology between the α2-M-R and LRP revealed that both proteins are identical [10]. The α2-M-R is a multifunctional cell-surface receptor of large size consisting of an external subunit (515 kDa) and a non-covalently attached transmembrane subunit (85 kDa) [8]. This receptor was found to bind and internalize diverse ligands [26]. The binding of these ligands seems to be regulated by a small receptor-associated protein (39 kDa) coeluting with the receptor during purification [9,19].

The interaction of purified α2-M with radiolabelled α2-M-R was studied in aqueous two-phase systems composed of 5% (w/w) PEG 6000, 7.5% (w/w) Dx T70 and buffer. Triton X-100 was included to keep the membrane receptor in solution. Due to the binding of detergent the α2-M-R partitioned into the top phase with a K-value of 2.19 (Fig. 4). Upon addition of increasing concentrations of α2-M-MA the K-value of the α2-M-R is decreased. In contrast, native α2-M had no effect on the partition of the receptor. It is known from the literature, that binding of α2-M-MA to the receptor

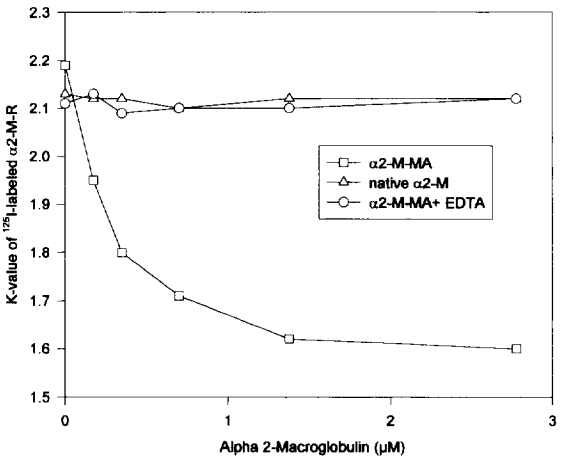

Fig. 4. Effect of native and transformed α2-M on partition of α2-M-R: 0.38 n*M* ^{125}I-labelled α2-M-R were partitioned in two-phase systems (2 g) composed of 5% PEG 6000, 7.5% Dx T70, 10 m*M* Hepes, 0.15 *M* NaCl, 5 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 0.05% Triton X-100, pH 7.4, and increasing concentrations of native (△) or transformed α2-M (□); (○) partition in the presence of α2-M-MA and 20 m*M* EDTA.

requires Ca^{2+}. This is also demonstrated by the effect of EDTA, which completely abolished the α2-M-MA–α2-M-R interaction. A number of ligands present in serum or plasma are bound to the receptor in addition to α2-M. According to the current knowledge, the diverse ligands are suggested to have distinct binding sites on the 515 kDa receptor subunit and ligand binding requires Ca^{2+}.

In Fig. 5 we analyzed in addition to α2-M the effect of serum components on partition of the α2-M-R. For calibration, the total concentration of α2-M in serum and methylamine-treated serum was equivalent to the purified α2-M-MA. As seen, methylamine-treated serum and surprisingly even normal serum, drastically changed the partition of the receptor. This alteration was more pronounced when compared with α2-M-MA. The slight difference between the partition curves of normal serum and methylamine-treated serum suggests that the strong alteration of the *K*-value is not attributable to α2-M. When the partition was conducted in the

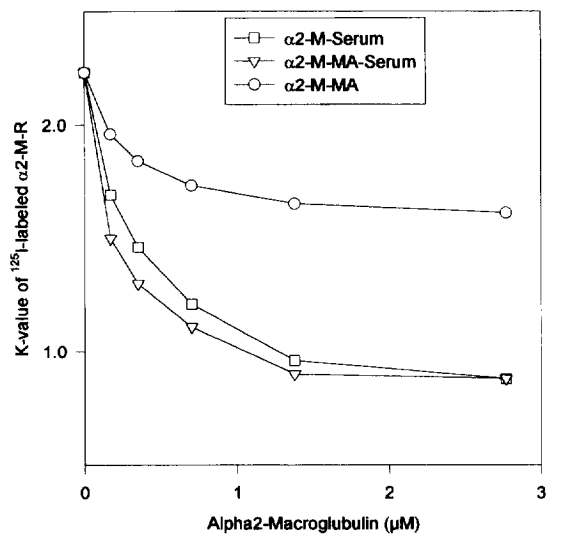

Fig. 5. Effect of of serum constituents on binding of α2-M to the receptor: 0.38 n*M* ^{125}I-labelled α2-M-R were partitioned in two-phase systems (2 g) composed of 5% PEG 6000, 7.5% Dx T70, 10 m*M* Hepes, 0.15 *M* NaCl, 5 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 0.05% Triton X-100, pH 7.4, and increasing concentrations of α2-M. (○) partition in the presence of α2-M-MA; (□) partition in the presence of fresh serum; (△) partition in the presence of methylamine-treated serum. The amounts of total α2-M in the different samples were adjusted to be equivalent.

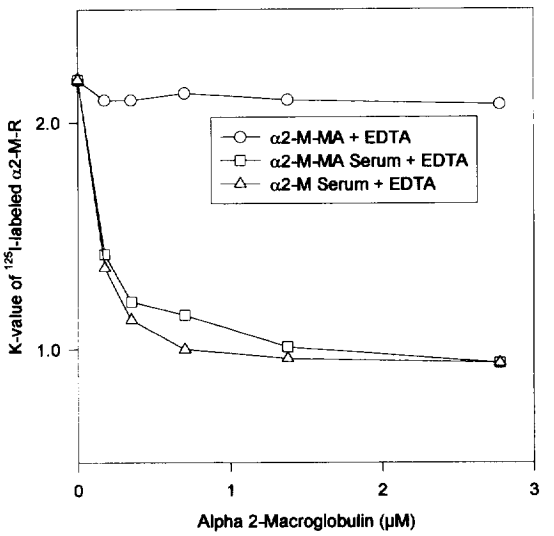

Fig. 6. Effect of EDTA on partition of α2-M receptor: 0.38 n*M* ^{125}I-labelled α2-M-R were partitioned in two-phase systems (2 g) composed of 5% PEG 6000, 7.5% Dx T70, 10 m*M* Hepes, 0.15 *M* NaCl, 5 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 0.05% Triton X-100, pH 7.4, and increasing concentrations of α2-M. (○) Partition in the presence of α2-M-MA plus 20 m*M* EDTA; (△) partition in the presence of fresh serum plus 20 m*M* EDTA; (□) partition in the presence of methylamine-treated serum plus 20 m*M* EDTA. The amounts of total α2-M in the different samples were adjusted to be equivalent.

presence of EDTA, the α2-M-MA–α2-M-R interaction was abolished, as assumed (Fig. 6). However, Ca^{2+}-complexation could not prevent the effect of serum on the partition of the receptor. Thus, these results suggest the existence of other serum components which effectively bind the receptor but independent of divalent cations. We looked for the possibility of unspecific effects on receptor binding by abundantly occurring serum components such as albumin and IgG. No effect on receptor partitioning was observed (data not shown).

To analyze competitive effects on ligand–α2-M-R interaction solid phase partition experiments were performed. Titer plates were coated with α2-M-R and the bound α2-M was analyzed by the ELISA technique. As demonstrated in Fig. 7, in the presence of Ca^{2+} α2-M-MA strongly binds to the immobilized receptor. When methylamine-treated serum was tested containing α2-M in comparable concentration, a reduced binding of α2-M-MA to the receptor was observed. This is due to a direct competition of serum components and α2-M-MA for receptor binding sites. The obviously low binding to the immobilized receptor of α2-M in normal serum was to be expected because more than 95% of total α2-M is native. Thus, these results suggest the existence of serum components which bind to the α2-M-R competitively to α2-M-MA but in a Ca^{2+}-independent manner. Such a serum component or components have not been described so far. The well known ligands of α2-M-R constituting serum proteins are all known to require Ca^{2+} for receptor interaction and are not competitive to α2-M-MA. Further investigations are directed to analyze the nature of these serum components.

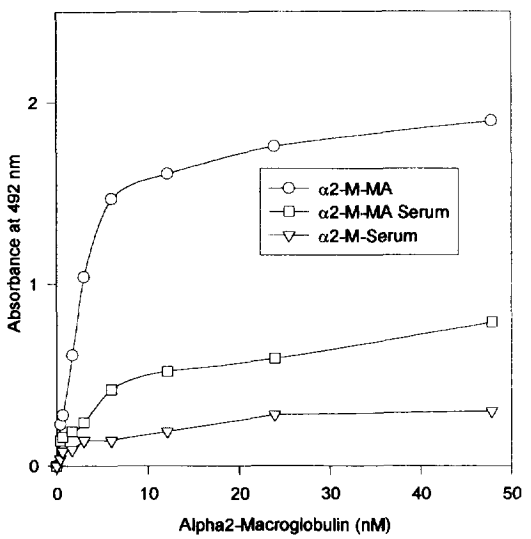

Fig. 7. Effect of serum components on binding of α2-M to the immobilized receptor. Titer plates coated with α2-M-R (2 μg/ml) were incubated with increasing amounts of α2-M-MA (○), serum (△) or methylamine-treated serum (□) for 1 h at 37°C. After washing the plates the bound α2-M was determined by anti-α2-M-POD in a colour reaction measured at 492 nm. The concentration of total α2-M in the different samples was adjusted to be identical.

Acknowledgments

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References

[1] A.J. Barrett and P.M. Starkey, Biochem. J., 133 (1973) 709.
[2] L. Sottrup-Jensen, J. Biol. Chem., 264 (1989) 11539.
[3] F. Van Leuven, P. Marynen, J.-J. Cassiman and H. Van den Berghe, Biochem. J., 203 (1982) 405.
[4] S.K. Moestrup, J. Gliemann and G. Pallesen, Cell Tissue Res., 269 (1992) 375.
[5] J. LaMarre, G.K. Wollenberg, S.L. Gonias and M.A. Hayes, Lab. Invest., 65 (1991) 3.
[6] W. Borth and T. Luger, J. Biol. Chem., 264, (1989) 5818.
[7] T. Selisko, J. Kratzsch and G. Birkenmeier, J. Clin. Endocrinol. Metab., 80 (1995) 585.
[8] J. Herz, U. Hamann, S. Rogne, O. Myklebost, H. Gausepohl and K.K. Stanley, EMBO J., 7 (1988) 4119.
[9] J. Herz, J.L. Goldstein, D.K. Strickland, Y.K. Ho and M.S. Brown, J. Biol. Chem., 266 (1991) 21232.
[10] D.K. Strickland, J.D. Ashcom, S. Williams, W.H. Burgess, M. Migliorini and W.S. Agraves, J. Biol. Chem., 265 (1990) 17401.
[11] R.C. Kowal, J. Herz, J.L. Goldstein, V. Esser, M. Brown, Proc. Natl. Acad. Sci. USA, 86 (1989) 5810.
[12] D.A. Chappell, G.L. Fry, M.A. Waknitz, P.-H. Iverius, S.E. Williams and D.K. Strickland, J. Biol. Chem., 267 (1992) 25764.
[13] A. Nykjaer, C.M. Petersen, B. Moller, P.H. Jensen, S.K. Moestrup, T.L. Holtet, M. Etzerodt, H.C. Thogersen, M. Munch, P.A. Andreasen and J. Gliemann, J. Biol. Chem., 267 (1992) 14543.

[14] K. Orth, E.L. Madison, M.-J. Gething, J.F. Sambrook and J. Herz, J. Biol. Chem., 89 (1992) 7426.
[15] G. Birkenmeier and T. Stigbrand, J. Immunol. Methods., 162 (1993) 59.
[16] P.-A. Albertsson, Partition of Cell Particles and Macromolecules, Wiley, New York, 3rd ed., 1986.
[17] H. Walter, D.E. Brooks and D. Fisher, Partitioning in Aqueous Two-Phase Systems. Theory, Methods, Uses and Applications to Biotechnology, Academic Press, Orlando, FL, 1985.
[18] G. Birkenmeier, M.A. Vijayalakshmi, T. Stigbrand and G. Kopperschläger, J. Chromatogr., 539 (1991) 267.
[19] J.D. Ashcom, S.E. Tiller, K. Dickerson, J.L. Cravens, W.S. Agraves and D.K. Strickland, J. Biol. Chem., 110 (1990) 1041.
[20] W.M. Hunter and F.C. Greenwood, Nature, 194 (1962) 495.
[21] S.L. Gonias, Exp. Hematol., 20 (1992) 302.
[22] G. Birkenmeier, L. Carlsson-Bostedt, V. Shanbhag, T. Kriegel, G. Kopperschläger, L. Sottrup-Jensen and T. Stigbrand, Eur. J. Biochem., 183 (1989) 239.
[23] A.F. Parisi and B.L. Vallee, Biochemistry, 9 (1970) 2421.
[24] R.J. Sundberg and R.B. Martin, Chem. Rev., 74 (1974) 471.
[25] A. Otto and G. Birkenmeier, J. Chromatogr., 644 (1993) 25.
[26] W. Borth, R.D. Feinman, S.L. Gonias, J.P. Quigley and D.K. Strickland (Editors) Biology of α2-Macroglobulin, Its Receptor, and Related Proteins, Ann. N.Y. Acad. Sci., The New York Academy of Sciences, New York, 1994, 737 pp.