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Inhibition of rat mast cell protease 1 by vitronectin

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Abstract

Rat mast cell protease 1 (RMCP-1) is a chymotrypsin-like serine protease specifically expressed by connective tissue-type mast cells. The enzyme is stored in the secretory granules in a macromolecular complex with heparin proteoglycan. In the present investigation it was shown that RMCP-1 is inhibited by vitronectin (VN), an RGD-containing adhesive glycoprotein with heparin-binding properties. RMCP-1 that had been separated from heparin proteoglycan was less susceptible to inhibition than RMCP-1 present in complex with heparin proteoglycan. Pre-incubation of VN with purified heparin partially blocked the RMCP-1 inhibiting activity of VN. Plasma VN had negligible RMCP-1-inhibiting activity. However, heat treatment of plasma VN, which is known to expose the heparin-binding domain, induced RMCP-1-inhibiting activity. Affinity chromatography on immobilized VN showed that RMCP-1 bound with high affinity to VN. The binding of RMCP-1 to VN was not heparin-dependent since free RMCP-1 bound with equal affinity to the immobilized VN as RMCP-1 present in complex with heparin. The inhibition of RMCP-1 by VN was shown to be reversible.

Key words: Vitronectin; Mast cell; Serine protease; Chymase; Heparin; Heparin-binding site

1. Introduction

Mast cells synthesize various inflammatory mediators, including histamine, chemotactic factors, proteoglycans, carboxypeptidases and serine proteases, that are stored in the secretory granules and are exocytosed following mast cell activation [1]. The mast cell secretory granule serine proteases constitute a family of related enzymes, including proteases of trypsin- (tryptases) and chymotrypsin-like (chymases) substrate specificities [2–6]. During the purification of rat mast cell protease 1 (RMCP-1), a chymotrypsin-like serine protease with thrombin-inactivating properties expressed by rat peritoneal mast cells, we observed that the protease is recovered from the cells as a macromolecular complex with heparin proteoglycans [7,8]. Heparin is a sulfated glycosaminoglycan displaying a high anionic charge density [9]. In the proteoglycan form, the heparin chains are covalently attached to a protein core. When RMCP-1 was separated from the heparin proteoglycan, the protease lost its ability to inactivate thrombin. Since RMCP-1 regained its thrombin-inactivating properties after reconstitution with heparin, we proposed that the association with heparin was important for the proteolytic activity of the enzyme. Polybrene, a polycationic heparin antagonist, was shown to inhibit RMCP-1 activity, further supporting the involvement of the heparin moiety of the RMCP-1/ heparin complex in the proteolytic activity of RMCP-1 [7].

Vitronectin (VN) is a multifunctional adhesive glycoprotein containing the RGD-sequence motif, implicated in the process of cellular adhesion mediated by integrins [10,11]. In addition, VN contains binding sites for various ligands such as heparin [10], collagen [12], plasminogen activator inhibitor type 1 [13] complement components [14] and thrombin/antithrombin complexes [15], as well as an acidic domain [16]. Some of these sites appear to be cryptic in the plasma form of VN but become exposed after unfolding of the molecule following treatment of VN by denaturing agents [17–19] or possibly by various physiological inducers [20,21] When the heparin-binding domain is exposed, VN gains potent heparin-neutralizing activity [22] and these findings encouraged us to assess whether VN, by interacting with the heparin moiety of the RMCP-1/heparin proteoglycan complex, had the capacity to inhibit the proteolytic activity of RMCP-1. In the present work we indeed show that VN inhibits RMCP-1 activity. It is, to our knowledge, the first report where protease inhibitory activity is attributed to VN.

2. Materials and methods

The chromogenic peptide substrate S-2586 was from Chromogenix (Mölndal, Sweden). Bovine chymotrypsin was a generous gift from I. Björk (Dept. Veterinary Medical Chemistry, Uppsala, Sweden). Unlabeled and [^{3}H]NAc-labeled pig mucosal heparins were kindly donated by Ulf Lindahl (Dept. Medical and Physiological Chemistry, Uppsala, Sweden). CNBr-activated Sepharose 4B and heparin–Sepharose were purchased from Pharmacia (Uppsala, Sweden). Purified VN (~2 mg) was coupled to ~2 ml of CNBr-activated Sepharose 4B according to the procedure described by the manufacturer.

Plasma VN, purified according to the method of Dahlbäck and Podack [23], was a gift from D.F. Mosher (Dept. Medicine, University of Wisconsin at Madison, USA). The method for purification utilizes mild chromatographic steps that do not significantly alter the native conformation of VN. Unfolded VN was purified according to the

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Abbreviations: RMCP, rat mast cell protease; VN, vitronectin; PBS, phosphate-buffered saline.

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method of Yatohgo et al. [24]. This method involves treatment of plasma VN with urea followed by affinity chromatography on heparin–Sepharose.

RMCP-1 present as a macromolecular complex with heparin proteoglycan was purified from rat peritoneal mast cells by anion-exchange chromatography on DEAE-Sephacel [7]. Free RMCP-1 was obtained after further purification on a Superdex 75 column as described [7].

RMCP-1 activities were measured in 96-well microtiter plates. RMCP-1 was diluted with PBS, 0.1% Triton X-100 to a final volume of 200 μl. Enzyme activity was detected after addition of 20 μl of the chromogenic chymotrypsin substrate S-2586 (4 mM in H_2O), and monitoring of the absorbance at 405 nm with a Titertek Multiscan spectrophotometer (Flow Laboratories). In the inhibition studies, RMCP-1 was pre-incubated for 5 min with VN before the addition of S-2586. Calculations of K_i values for the inhibition of RMCP-1 by VN were carried out as described [25].

Affinity chromatography of RMCP-1 on VN–Sepharose was performed on a column (~ 0.6 ml) equilibrated with PBS, 0.1% Triton X-100. Samples of RMCP-1, RMCP-1/heparin complexes or ^{3}H-labeled heparin were applied to the column, followed by washing of the column with 2 × 1 ml of equilibrating buffer. The column was then eluted stepwise with PBS, 0.1% Triton X-100 containing 0.3, 0.5, 1, 2 and 3 M NaCl, respectively (2 × 1 ml of each buffer). The eluted fractions were subsequently analyzed for RMCP-1 activity or ^{3}H radioactivity.

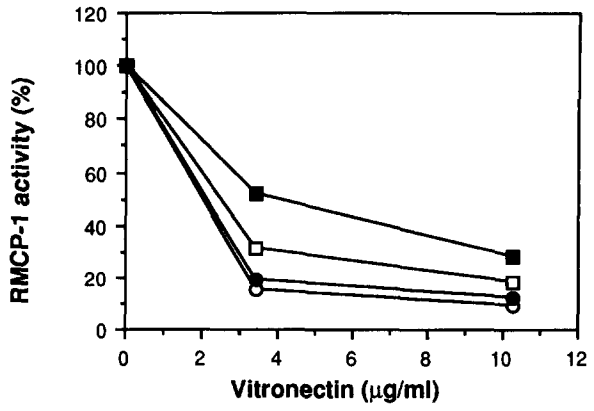

Fig. 2. Blocking of RMCP-1-inhibiting activity of VN by heparin. VN at the concentrations indicated was pre-incubated with 5 μg/ml (●), 20 μg/ml (□) or 100 μg/ml (■) of heparin (standard pig mucosal heparin), or without added heparin (○). After 15 min, RMCP-1/heparin proteoglycan complex (~0.2 μg of RMCP-1/ml) was added to the incubation mixtures, and, after an additional 5 min, residual RMCP-1 activity was determined with the chromogenic substrate S-2586.

3. Results

RMCP-1 has previously been shown to be recovered in a macromolecular complex with heparin proteoglycan [7]. Incubation of complex-bound RMCP-1 with unfolded VN resulted in inhibition of the protease, as measured by the cleavage of the chromogenic chymotrypsin substrate S-2586 (Fig. 1). VN at similar concentrations also inhibited the ability of the complex-bound RMCP-1 to inactivate thrombin (not shown). Free RMCP-1, separated from the heparin proteoglycan by gel-filtration in high salt (see [7]), was also inhibited by VN. However, higher VN concentrations were required for inhibition of the free protease. Purified chymotrypsin was not inhibited by VN (Fig. 1). Calculations based on the data shown in Fig. 1 showed that the K_i value for the inhibition of complex-bound RMCP-1 was 3.6 ± 1.2 nM, whereas the K_i for the inhibition of free RMCP-1 was 2 ± 7 nM, thus indicating that the inhibition of complex-bound RMCP-1 by VN is ~ 7-fold more effective than the inhibition of free RMCP-1. The calculations of K_i values are based on the assumption that VN is present in the monomeric form. However, VN has a tendency to multimerize [18], and therefore the K_i values should be taken as approximate.

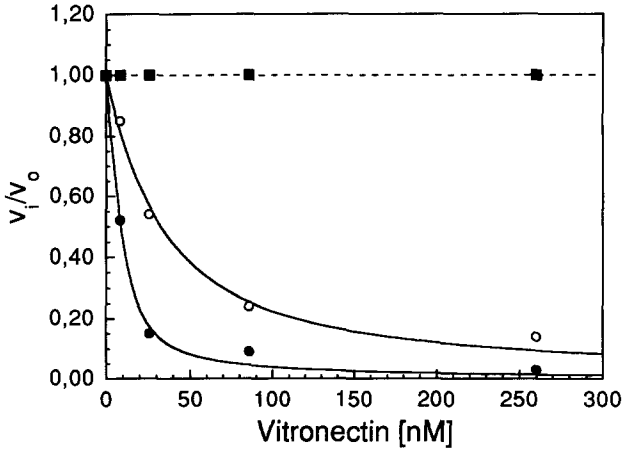

Fig. 1. Inhibition of RMCP-1 and chymotrypsin by VN. Free RMCP-1 (~0.2 μg/ml; ○), RMCP-1/heparin proteoglycan complex (~0.2 μg of RMCP-1/ml; ●) or chymotrypsin (~ 0.2 μg/ml; ■) were pre-incubated for 5 min with VN at the concentrations indicated. Subsequently, residual protease activity was determined after addition of the chromogenic substrate S-2586. The solid lines are the fitted curves, generated using the best estimates calculated by non-linear regression analysis.

The finding that RMCP-1, complex-bound to heparin, is more readily inhibited by VN than free RMCP-1 suggests the involvement of the RMCP-1-associated heparin proteoglycan in the inhibition mechanism. As VN contains a heparin-binding domain it appeared likely that VN interacts with the heparin moiety of the RMCP-1/heparin complex. This possibility was tested in experiments where VN was pre-incubated with standard pig mucosal heparin before addition to the RMCP-1/heparin proteoglycan complex. The obtained results showed that exogeneous heparin partially blocked the protease-inhibitory activity of VN in a dose-dependent manner (Fig. 2).

The above findings indicated that the heparin-binding domain of VN may participate in the inhibition of RMCP-1. Plasma VN represents a form of the molecule where the heparin-binding site is buried. However, various treatments of VN, such as treatment with 8 M urea or heat treatment of the molecule, are known to expose the heparin-binding domain. To further study the inhibition mechanism, the RMCP-1-inhibiting activities of plasma VN and unfolded VN were compared. Plasma VN was shown to be a poor inhibitor of RMCP-1

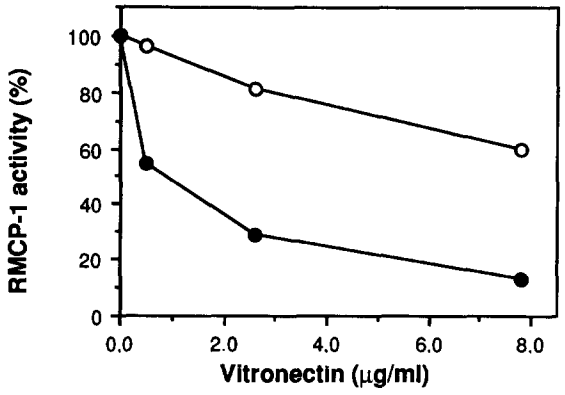

Fig. 3. Inhibition of RMCP-1 by plasma VN. RMCP-1/heparin proteoglycan complex (~0.2 μg of RMCP-1/ml) was incubated with plasma VN (○) or plasma VN that had been heated at 95°C for 2 min (●). After 5 min, residual RMCP-1 activity was determined after addition of the chromogenic substrate S-2586.

(Fig. 3). In contrast, plasma VN that had been heated at 95°C for 2 min inhibited RMCP-1 effectively (Fig. 3).

Experiments were conducted to assess whether the inhibition of RMCP-1 by VN was reversible. RMCP-1/heparin proteoglycan complex was incubated at 0.15 M NaCl with VN at a concentration sufficient to achieve essentially total inhibition of the protease (Fig. 4A). Subsequently, NaCl was added both to control incubations of RMCP-1/heparin proteoglycan and to incubations of RMCP-1/heparin proteoglycan containing VN, yielding final NaCl concentrations of 0.3 M and 0.5 M, respectively (Fig. 4B). The higher salt concentrations resulted in a slight inhibition of the RMCP-1 present in the control incubations. Moreover, at 0.3 M NaCl, partial recovery of RMCP-1 activity from incubations containing RMCP-1/heparin proteoglycans and VN was observed. At 0.5 M NaCl, total recovery of the RMCP-1 activity previously inhibited by VN was obtained, demonstrating that the inhibition of RMCP-1 by VN is reversible (Fig. 4B).

To further study the interaction of RMCP-1 with VN, RMCP-1 was subjected to affinity chromatography on immobilized VN (VN–Sepharose). RMCP-1 present in the macromolecular complex with heparin proteoglycan bound strongly to the affinity matrix. Stepwise elution of the column showed that most of the RMCP-1 activity was eluted at 1–2 M NaCl (Fig. 5A). Free RMCP-1, separated from the heparin proteoglycan displayed similar affinity for the immobilized VN as complex-bound RMCP-1 (Fig. 5A). Affinity chromatography of ^{3}H-labeled heparin chains on the VN–Sepharose column showed that the radiolabeled heparin bound to the column and eluted at ~0.5 M NaCl. When a mixture of ^{3}H-labeled heparin and RMCP-1 (separated from the heparin proteoglycan) was applied to the immobilized VN, the radiolabeled heparin eluted at ~0.5 M NaCl, whereas the RMCP-1 activity showed a similar elution profile as the free RMCP-1 (Fig. 5B).

4. Discussion

RMCP-1 is a highly basic protein with a net positive charge of +18 [2]. The high positive charge displayed by the protease suggests the potential of interaction with various acidic molecules. Indeed, RMCP-1 is stored in the granules in a macromolecular complex with heparin proteoglycan [7,26], and is released from the mast cells bound to the heparin proteoglycan [27]. The association of RMCP-1 with heparin appears to influence the activity of the enzyme. Yurt and Austen [26] and Sayama et al. [28] have reported that heparin reduces the activity of mast cell chymase. In contrast, Gervasoni et al. [29] have shown that interaction of the mast cell chymase with heparin is necessary for the degradation of anaphylatoxin C3a. Further, we have recently shown that the association of RMCP-1 [7,8] and also mouse mast cell chymase [30] with heparin is required for the inactivation of thrombin. As thrombin is known to interact with heparin, we proposed a mechanism where thrombin, by binding to the RMCP-1-associated heparin proteogly-

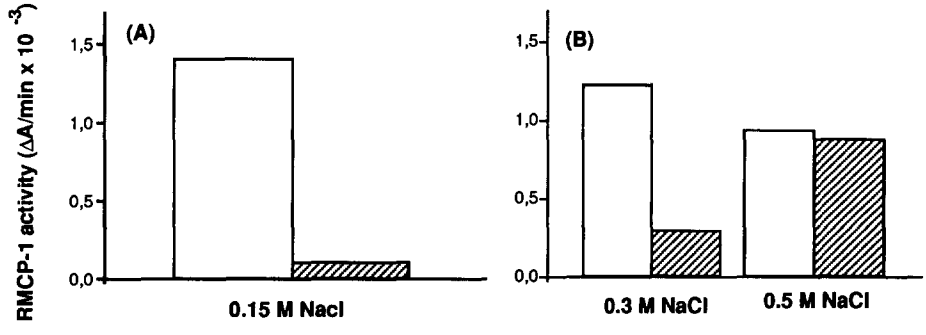

Fig. 4. Reversibility of the inhibition of RMCP-1 by VN. (A) RMCP-1/heparin proteoglycan complex (~0.2 μg of RMCP-1/ml) was incubated for 5 min in the absence (open bar) or presence (hatched bar) of 2 μg of VN, followed by determination of residual RMCP-1 activity with the chromogenic substrate S-2586. (B) RMCP-1/heparin proteoglycan complex (~0.2 μg of RMCP-1/ml) was incubated for 5 min in the absence (open bars) or presence (hatched bars) of 2 μg of VN. After 5 min, NaCl from a stock solution containing 2 M NaCl was added to the incubation mixtures, yielding final NaCl concentrations of 0.3 M and 0.5 M, respectively. RMCP-1 activity was determined after 5 min.

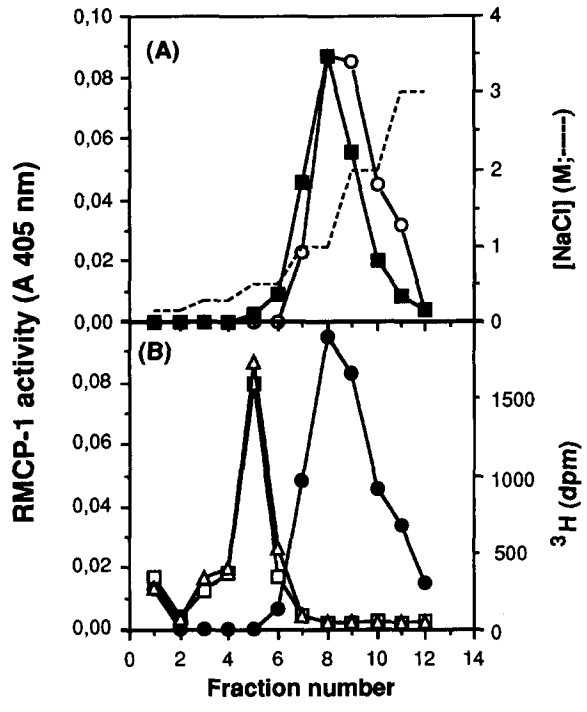

Fig. 5. Affinity chromatography on VN–Sepharose. (A) Samples of RMCP-1/heparin proteoglycan complex (~ 0.5 μg of RMCP-1; ■) or free RMCP-1 (~0.5 μg; ○) were applied to a column of VN–Sepharose. The column was eluted with PBS,0.1% Triton X-100 supplemented with NaCl at the concentrations indicated. Eluted fractions were analyzed for RMCP-1 activity. (B) A sample of RMCP-1 (~0.5 μg) was mixed with [^{3}H]heparin (4,000 dpm) and was applied to the VN–Sepharose. Eluted fractions were analyzed for RMCP-1 activity (●) or ^{3}H radioactivity (□). (△) Affinity chromatography of [^{3}H]heparin (4,000 dpm) on VN–Sepharose.

can, is brought in close contact with the chymase, thereby facilitating proteolysis. Furthermore, we found that Polybrene, a polycationic heparin antagonist, inhibited the inactivation of thrombin by RMCP-1, presumably by binding to the heparin moiety of the RMCP-1/heparin proteoglycan complex [7]. We therefore hypothesized that naturally occurring heparin antagonists may have RMCP-1-inhibiting activity. Indeed, in the present report we show that VN inhibits RMCP-1. The mechanism of inhibition seemed to involve the heparin-binding site of VN since free RMCP-1, separated from the heparin proteoglycan, was less susceptible to inhibition by VN. This notion is further supported by the experiments where pre-incubation of VN with heparin partially blocked its ability to inhibit RMCP-1.

It is important to note that it is the unfolded form of VN that exerts the RMCP-1-inhibiting activity, as well as several other reported activities associated with the protein. Plasma VN was shown to have very low RMCP-1-inhibiting activity, consistent with only a minor portion of the circulating VN molecules being present in the unfolded form [17,31]. However, heat treatment of the plasma VN, which is known to unfold the protein [18], induced RMCP-1-inhibiting activity. The unfolding of VN exposes the heparin-binding domain along with other cryptic domains, and it thus appears that the unfolding process is necessary to achieve the RMCP-1-inhibiting activity. The unfolding procedure can also occur by exposure to various physiological agents, e.g. heparin [32], antithrombin/thrombin complexes [20] and complement complexes [21]. Thus, it appears that many physiological functions of VN, particularly those involving heparin-binding [18,19], can be modulated.

Affinity chromatography on immobilized (unfolded) VN showed that RMCP-1 exhibits high affinity for VN. The binding to VN did not appear to be mediated by the heparin moiety of the RMCP-1/heparin proteoglycan complex since free RMCP-1 bound equally well to VN–Sepharose as RMCP-1 present in complex with heparin proteoglycan. In fact, free RMCP-1 showed higher affinity for VN than did purified heparin. As RMCP-1 carries a high net positive charge [2], interaction with a negatively charged domain of VN would explain the binding of RMCP-1 to VN. Such an acidic domain of VN is located in the N-terminal region of the molecule close to the RGD sequence [16], and it is thus possible that this area of VN has the ability to bind to positively charged regions of RMCP-1. Since our results indicate that also the heparin-binding domain of VN is involved in the inhibition of RMCP-1 (see above), it is thus possible that optimal inhibition of RMCP-1 requires interaction of both the heparin-binding domain of VN with the heparin moiety of the RMCP-1/heparin proteoglycan complex, and interaction of the acidic domain of VN directly with RMCP-1. It is worth noting that 1–2 M NaCl was required for dissociation of RMCP-1 from the VN–Sepharose whereas 0.5 M NaCl was sufficient both for the dissociation of heparin from the VN–Sepharose and for the reversal of the inhibition of RMCP-1 (present in complex with heparin) by VN.

Due to the multidomain structure of VN the protein is thought to have a variety of biological functions. The RGD sequence mediates the integrin-dependent cell adhesion to VN [11]. Moreover, the interaction of VN with heparin [18,19], thrombin/serpin complexes and plasminogen activator inhibitor type 1 [33,34] indicates a role for VN in the regulation of hemostasis. There is also evidence for the participation of VN in the immune defence. Specific interactions between VN and bacteria have been observed [35] and it is thought that VN plays a role in the initial adherence of bacteria to host cells. VN has also been shown to inhibit the complement system by interfering with the assembly of the membrane attack complex [14]. The involvement of VN in the inflammatory response is further suggested by the immunohistochemical identification of VN at inflammatory sites [33,34,36,37]. The biological function of the mast cell chymases is uncertain. However, various reported activities for the proteases [29,30,38–40] indicate that they are involved in

inflammatory reactions. Considering the vast amounts of RMCP-1 present in the mast cell granules [27], it appears likely that a regulatory mechanism capable of neutralizing RMCP-1, released after mast cell activation, is important. The present report identifies VN as a potent inhibitor of RMCP-1. Since VN appears to be present at inflammatory sites, the inhibition of RMCP-1 by VN may thus be of physiological significance in the regulation of the inflammatory response.

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References

[1] Stevens, R.L. and Austen, K.F. (1989) Immunol. Today 10, 381–386.
[2] Le Trong, H., Parmelee, D.C., Walsh, K.A., Neurath, H. and Woodbury, R.G. (1987) Biochemistry 26, 6988–6994.
[3] Schechter, N.M., Choi, J.K., Slavin, D.A., Deresienski, D.T., Sayama, S., Dong, G., Lavker, R.M., Proud, D. and Lazarus, G.S. (1986) J. Immunol. 137, 962–970.
[4] Huang, R., Blom, T. and Hellman, L. (1991) Eur. J. Immunol. 21, 1611–1621.
[5] Reynolds, D.S., Stevens, R.L., Lane, W.S., Carr, M.H., Austen, K.F. and Serafin, W.E. (1990) Proc. Natl. Acad. Sci. USA 87, 3230–3234.
[6] Miller, J.S., Westin, E.H. and Schwartz, L.B. (1989) J. Clin. Invest. 84, 1188–1195.
[7] Pejler, G., Söderström, K. and Karlström, A. (1994) Biochem. J. 299, 507–513.
[8] Pejler, G. and Maccarana, M. (1994) J. Biol. Chem. (in press).
[9] Kjellén, L. and Lindahl, U. (1991) Annu. Rev. Biochem. 60, 443–475.
[10] Suzuki, S., Pierschbacher, M.D., Hayman, E.G., Nguyen, K., Ohgren, Y. and Ruoslahti, E. (1984) J. Biol. Chem. 259, 15307–15314.
[11] Pytela, R., Pierschbacher, M.D. and Ruoslahti, E. (1985) Proc. Natl. Acad. Sci. USA 82, 5766–5777.
[12] Gebb, C., Hayman, E.G., Engvall, E. and Ruoslahti, E. (1986) J. Biol. Chem 261, 16698–16703.
[13] Declerck, P.J., De Mol, M., Alessi, M.-C., Baudner, S., Paques, E.-P., Preissner, K.T., Müller-Berghaus, G. and Collen, D. (1988) J. Biol. Chem. 263, 15454–15461.
[14] Podack, E.R. and Müller-Eberhard, J. (1979) J. Biol. Chem 254, 9908–9914.
[15] Ill, C.R. and Ruoslahti, E. (1985) J. Biol. Chem. 260, 15610–15615.
[16] Jenne, D., Hille, A., Stanley, K.K. and Huttner, W.B. (1989) Eur. J. Biochem. 185, 391–395.
[17] Barnes, D.W., Reing, E. and Amos, G. (1985) J. Biol. Chem. 260, 9117–9122.
[18] Stockmann, A., Hess, S., Declerck, P., Timpl, R. and Preissner, K.T. (1993) J. Biol. Chem. 268, 22874–22882.
[19] Bittorf, S.V., Williams, E.C. and Mosher, D.F. (1993) J. Biol. Chem. 268, 24838–24846.
[20] Tomasini, B.R. and Mosher, D.F. (1988) Blood 72, 903–912.
[21] Høgåsen, K., Mollnes, R.E. and Harboe, M (1992) J. Biol. Chem. 267, 23076–23082.
[22] Lane, D.A., Flynn, A.M., Pejler, G., Lindahl, U., Choay, J. and Preissner, K.T. (1987) J. Biol. Chem. 262, 16343–16348.
[23] Dahlbäck, B. and Podack, E.R. (1985) Biochemistry 24, 2368–2374.
[24] Yatohgo, T., Izumi, M., Kashiwagi, H. and Hayashi, M. (1988) Cell Struct. Funct. 13, 281–292.
[25] Bieth, J.G. (1974) Bayer Symposium V, 463–469.
[26] Yurt, R.W. and Austen, K.F. (1977) J. Exp. Med. 146, 1405–1419.
[27] Schwartz, L.B., Riedel, C., Caulfield, J.P., Wasserman, S.I. and Austen, K.F. (1981) J. Immunol. 126, 2071–2078.
[28] Sayama, S., Iozzo, R.V., Lazarus, G.S. and Schechter, N.M. (1987) J. Biol. Chem. 262, 6808–6815.
[29] Gervasoni Jr, J.E., Conrad, D.H., Hugli, T.E., Schwartz, L.B. and Ruddy, S. (1986) J. Immunol. 136, 285–292.
[31] Izumi, M., Yamada, K.M. and Hayashi, M. (1989) Biochim. Biophys. Acta 990, 101–108.
[32] Hildebrand, A., Preissner, K.T., Müller-Berghaus, G. and Teschemacher, H. (1989) J. Biol. Chem. 264, 15429–15434.
[33] Tomasini, B.R. and Mosher, D.F. (1991) Prog. Hemost. Thromb. vol. 10 (Coller, B. ed.) pp. 269–305, Saunders, Philadelphia.
[34] Preissner, K.T. (1991) Annu. Rev. Cell Biol. 7, 275–310.
[35] Chhatwal, G.S., Preissner, K.T., Müller-Berghaus, G. and Blobel, H. (1987) Infect. Immun. 55, 1878–1883.
[36] Podor, T.J., Joshua, P., Butcher, M., Seiffert, D., Loskutoff, D. and Gauldie, J. (1992) Ann. NY Acad. Sci. 667, 173–177.
[37] Dahlbäck, K., Wulf, H.-C. and Dahlbäck, B. (1993) J. Invest. Dermatol. 100, 166–170.
[38] Mizutani, H., Schechter, N., Lazarus, G., Black, R.A. and Kupper, T.S. (1991) J. Exp. Med. 174, 821–825.
[39] Katunuma, N. and Kido, H. (1988) J. Cell. Biochem. 38, 291–301.
[40] Reilly, C.F., Tewksbury, D.A., Schechter, N.M. and Travis, J. (1982) J. Biol. Chem. 257, 8619–8622.