High-molecular-weight kininogen is a binding protein for tissue prokallikrein

Armin Raab, Michael Kemme*

Institute for Biochemistry, Darmstadt University of Technology, Petersenstr. 22, 64287 Darmstadt, Germany

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Abstract Human tissue prokallikrein, a zymogen of the kallikrein-kinin system, circulates in plasma bound to neutrophils. Because plasma kininogens contribute to the assembly of kinin-generating components on blood cells, these proteins were assessed for their ability to complex the kallikrein precursor. Using ligand blot and direct binding assays, biotinylated prokallikrein was found to bind only to high-molecular-weight kininogen and not to the low-molecular-weight form. The interaction was specific, reversible, and saturable yielding an estimated dissociation constant $K_D = 690$ nM and a 1:1 stoichiometry. Specific kininogen binding of tissue prokallikrein also occurred at physiological plasma protein concentrations. These results provide the first evidence for a novel function of high-molecular-weight kininogen as a binding protein for tissue prokallikrein that could serve to localize the kallikrein precursor on the neutrophil surface.

Key words: Binding protein; Kininogen; Tissue prokallikrein; Zymogen

1. Introduction

Human tissue prokallikrein, the zymogen of the serine proteinase tissue kallikrein (EC 3.4.21.35), has been implicated in acute inflammatory reactions, and all components of the prokallikrein-driven cascade system, i.e. tissue kallikrein, kininogens, and kinin peptides have been found in inflammatory effusions [1]. Abnormal levels of these factors in synovial fluid from inflamed joints and in bronchoalveolar lavage fluid from lower airways are associated with the pathogenesis of arthritis and asthma [2,3]. The abundance of tissue kallikrein is regulated at the levels of transcription [4] and posttranslation, the latter most likely through site-directed activation of prokallikrein [5]. This 245-amino-acid zymogen precursor is converted into the active enzyme by limited proteolysis of a single peptide bond at position 7 to release mature tissue kallikrein which, in turn, can liberate the kinin decapeptide kallidin (lysyl-bradykinin) from high- or low-molecular-weight kininogen (HK, LK) [6]. Kinins are locally active hormones that, by binding to specific receptors at target cells, regulate vascular permeability, induce neutrophil chemotaxis, and mediate inflammatory cascades [7]. Gene expression of the kinin-forming kallikrein precursor occurs in multiple tissues and exocrine glands of the human body [7], but in the circulation, biosynthesis of prokallikrein mRNA has been demonstrated in human neutrophils only [8]. Recently, direct evidence for the presence of immunoreactive tissue prokallikrein in cytoplasmic granules of circulating neutrophils has been reported [5], however, a greater amount of the zymogen was associated into punctate clusters occupying the external surface of the neutrophil membrane [9]. Tissue prokallikrein docks to the plasma membrane via specific cell-attachment sites [10], but the structural identity of the putative zymogen acceptor molecule(s) is still unknown.

Similar clustered patterns of neutrophil-bound proteins have been reported for HK and LK present in patchy spots on the outer surface of the neutrophil membrane [11]. Both kininogens are multifunctional plasma proteins with a common heavy chain and kinin moiety, respectively, but LK is devoid of the specific light chain sequences that reside in HK [7]. Circulating neutrophils contain a considerable number of kininogen-binding sites which may be compromised of one or more candidate proteins. Recently, the leukocyte integrin Mac-1 (CD11b/18) has been shown to serve as a major attachment site for HK requiring a zinc-dependent interaction with specific domains of the heavy and light chain of HK, respectively [12]. The functional significance of neutrophil-bound kininogens is not yet fully delineated. Cell surface-anchored kininogens might act as docking proteins for additional components of the kallikrein-kinin system to trigger the kinin-forming pathway which has already been established for bimolecular complexes between HK and plasma prokallikrein on the membrane of endothelial cells [13,14]. Therefore, we have postulated a similar local assembly of tissue prokallikrein and kininogens on the surface of human neutrophils that could contribute through the release of kinins to the diapedesis of neutrophils during the inflammatory response.

Since specific acceptor molecules are required for the docking of tissue prokallikrein to neutrophils [10], it is of particular interest to identify prokallikrein-binding proteins and their potential relationship to the zymogen-binding sites on neutrophil membranes. In this study, we have used recombinant tissue prokallikrein and purified kininogens to investigate their possible interactions by direct binding and ligand blotting assays. In addition, human plasma was screened for proteins that specifically bind to tissue prokallikrein.

*Corresponding author. Fax: (49)-6151-16-5399.
E-mail: mkemme@pop.tu-darmstadt.de

Abbreviations: BSA (HSA), bovine (human) serum albumin; ELISA, enzyme-linked immunosorbent assay; HK, high-molecular-weight kininogen; LK, low-molecular-weight kininogen; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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2. Materials and methods

2.1. Materials

Purified single-chain HK and LK were purchased from Calbiochem (Bad Soden, Germany). Human serum albumin (HSA) and HK-deficient plasma (Fitzgerald trait) were obtained from Sigma (Deisenhofen, Germany). Normal plasma from healthy donors was collected by two centrifugation steps ($200\times g$, $10\,000\times g$, 10 min each) from whole blood immediately adjusted to 5.4 mM EDTA, 0.4 mM Pefabloc SC, and 10 μM E-64 (Roche Diagnostics, Mannheim, Germany). Preparation of recombinant human tissue prokallikrein, which was produced in insect cells using the baculovirus expression system, and conversion of the purified proenzyme to mature kallikrein followed established protocols [6]. Purified prokallikrein, mature kallikrein, and anti-TproK antibodies directed against the recombinant zymogen were biotinylated with EZ-Link biotin hydrazide (Pierce, Rockford, IL, USA) according to the manufacturer's recommendations. The degree of biotinylation was verified by an indirect ELISA using wells coated with various dilutions of the labeled protein and biotin-BSA (Sigma) as standards, respectively. Probing occurred with horseradish peroxidase-conjugated streptavidin (Dianova, Hamburg, Germany) and 2,2′-azino-bis(3-ethylbenzthiazoline)-sulfonate (ABTS, Roche Diagnostics).

2.2. Dot blot analysis

Samples were added to a grid pattern on a nitrocellulose membrane (BA 83, Schleicher and Schüll, Dassel, Germany) and air dried for 2 h at 4°C. After blocking free sites overnight with 5% (w/v) BSA in TBST-Ca (10 mM Tris–HCl, 150 mM NaCl, 2 mM $CaCl_2$, 0.05% Tween 20, pH 8), the dot blot was incubated with 0.5 μg/ml (15 nM) biotinylated prokallikrein in blocking buffer in the presence or absence of 50 μM Zn^{2+} with or without competitor for 2 h at room temperature. The membrane was washed three times in TBST-Ca and the bound ligand detected with streptavidin-peroxidase for 1 h, followed by the chemiluminescence system (ECL, Amersham Pharmacia, Buckinghamshire, England).

2.3. Ligand blotting

Samples were treated at 42°C for 30 min in buffer containing 0.5% SDS, 100 mM Tris–HCl, pH 6.8, 25% glycerol, but no reducing agents. After separation by SDS–PAGE, proteins were visualized with Coomassie blue R-250 or transferred to BA 83 nitrocellulose membranes by the semi-dry technique. Following incubation in 20 μg/ml (0.6 μM) recombinant prokallikrein, bound ligand was probed with biotinylated anti-TproK antibodies (800 ng/ml) and the subsequent streptavidin-peroxidase/ECL reaction.

2.4. Quantification of kininogen-bound biotin-prokallikrein

A direct binding assay on HK-coated (250 ng/well), 96-well microtiter plates was performed at 37°C in triplicate. Wells were blocked with 2% BSA in PBS for 1 h and then incubated for 2 h with serial dilutions of biotin-prokallikrein (starting concentration 67 μg/ml = 1.9 μM) and biotin-kallikrein (starting concentration 64 μg/ml = 1.9 μM), respectively, both dissolved in PBS containing 1% BSA. After washing with 0.05% Tween 20 in PBS, detection of bound proteins occurred with streptavidin-peroxidase for 2 h, followed by the ABTS substrate reaction for 30 min. The change in absorbance was monitored at 405 nm using a 340 ATTC ELISA reader (SLT Labinstruments, Crailsheim, Germany). To convert the absorbance at each amount of bound prokallikrein to pmoles zymogen bound, standard curves were monitored by an indirect ELISA using microtiter plates coated with serial dilutions of biotinylated prokallikrein. Non-specific binding was determined by the level of binding seen with mature kallikrein. Equilibrium binding data were analyzed by linear regression according to the method of Scatchard [15].

3. Results

In view of the fact that human tissue prokallikrein circulates in plasma predominantly complexed with neutrophils [9], initial experiments were performed to determine if cell surface docking of prokallikrein can be mediated by plasma kininogens. Using dot blot assays, purified HK and LK were compared for their binding properties towards biotinylated prokallikrein (Fig. 1A). Only HK and a positive method control with prokallikrein-specific antiserum displayed high affinity for nanomolar biotin-prokallikrein. No interaction was observed with LK demonstrating that the prokallikrein-binding site is comprised by the unique light chain of HK. Incubation in the presence of a 50-fold molar excess of unlabeled kallikrein precursor significantly reduced the binding of biotinylated prokallikrein. This result indicated not only the reversibility of the complex formation but also the specificity of prokallikrein association to HK. The specificity of the test system was further confirmed by an unrelated protein, human serum albumin (HSA), which failed to show a binding signal. In order to establish the optimal conditions for biotin-labeled prokallikrein reacting with HK, studies were carried out to determine the influence of zinc ions on the interaction between HK and the kallikrein zymogen (Fig. 1B). The amount of biotin-prokallikrein bound to HK in the presence of 50 μM Zn^{2+} was not significantly different from the level of binding in the absence of Zn^{2+}, thus demonstrating that zinc ions had no effect on the complex formation of prokallikrein and HK.

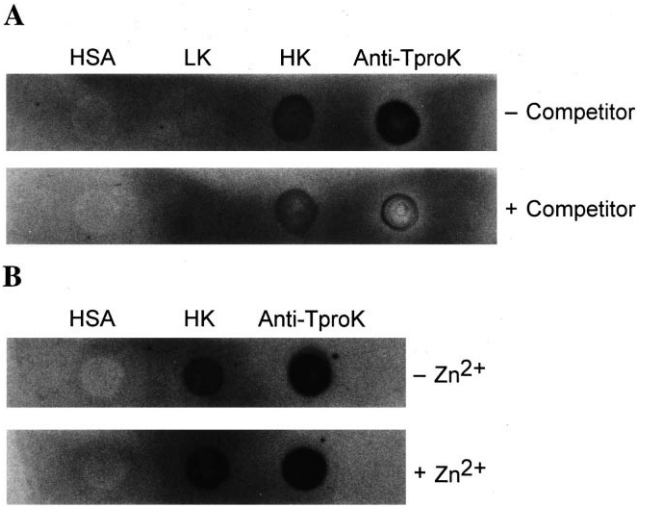

Fig. 1. Dot blot analysis of tissue prokallikrein binding by various plasma proteins. Immobilized proteins (2 μg per spot) and 2 μl anti-prokallikrein antiserum (anti-TproK, 1:500 dilution) were probed with biotinylated prokallikrein in the presence of a 50-fold molar excess of unlabeled prokallikrein (competitor) (A), and in the presence of 50 μM zinc ions (B).

The number and affinity of tissue prokallikrein-binding sites on HK were then determined at equilibrium as a function of ligand concentration (Fig. 2A). When increasing amounts of biotinylated prokallikrein and mature kallikrein, respectively, were added to immobilized HK at 37°C, kininogen bound the kallikrein precursor most efficiently in a dose-dependent manner whereas the affinity to the mature enzyme was significantly lower. Specific binding of prokallikrein reached saturation at 4 nM of the ligand, which represented approximately 0.4% bound of the total kallikrein precursor present. To quantify zymogen affinity, binding of the kallikrein portion was subtracted from total bound zymogen at each HK concentration to obtain the contribution of the propart of prokallikrein associated to kininogen. Analysis of the data by the graphical method of Scatchard (Fig. 2B) indicated that biotin-prokallikrein bound with moderate affinity to HK yielding an apparent dissociation constant K_D of 690 nM. The stoichiometry

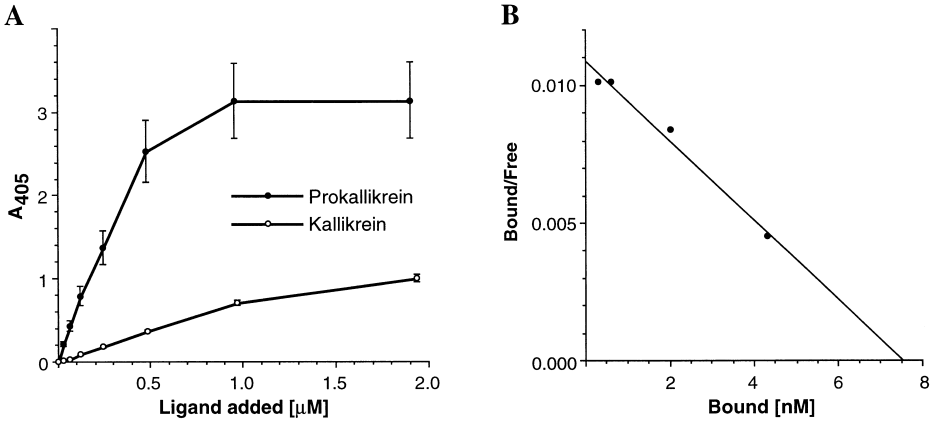

Fig. 2. Direct binding of tissue prokallikrein to immobilized HK. The ability of HK to interact with increasing concentrations of biotinylated prokallikrein and kallikrein, respectively, was determined by the streptavidin-peroxidase system (A). The best-fit data of the ratio of bound/free versus bound prokallikrein are presented in a Scatchard plot (B).

of binding in the bimolecular complex was found to be 1:1 as calculated from the total number of bound ligand (B_{max} = 1.5 pmol/well) which was equivalent to the amount of immobilized HK.

Since tissue prokallikrein appeared to bind selectively to HK, a ligand blot was performed to assess prokallikrein-binding activity under physiological protein concentrations in human plasma (Fig. 3). SDS-denatured proteins were subjected to Western blotting after electrophoretic separation and prokallikrein-binding proteins were then visualized by the formation of a binding protein–prokallikrein–antibody complex on the blot. In the complex mixture of plasma proteins, this assay detected a specific prominent band at 116 kDa in normal human plasma, similar to the band obtained with commercially purified HK. As a method control, the HK binding could not be identified in kininogen-deficient plasma. Trace reactivity of two other proteins in the 80–90 kDa range is seen in normal plasma and kininogen-deficient plasma, respectively. These additional bands might point to the existence of further prokallikrein-binding proteins yet unidentified. The intense band in the high-molecular-weight range (>120 kDa) of both plasma samples reflects an artifact of biotinylated anti-prokallikrein antibody as this reactivity was also observed in the absence of the prokallikrein ligand (data not shown).

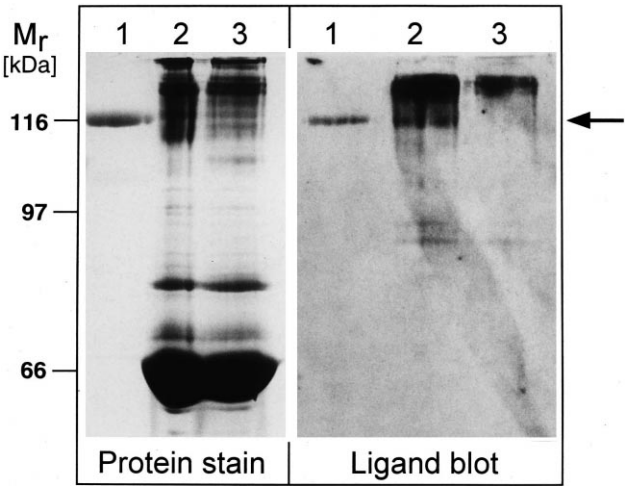

Fig. 3. Detection of a prokallikrein-binding protein in human plasma. Purified HK (lane 1, 2 μg), plasma (lane 2, 50 μg) and HK-deficient plasma (lane 3, 50 μg) were resolved by 10% SDS–PAGE under non-reducing conditions. Proteins were visualized by Coomassie staining (left panel) and ligand blotting in the presence of recombinant human tissue prokallikrein (right panel). The arrow indicates the relative position of HK. Molecular masses of marker proteins are given on the left.

4. Discussion

The study described above is the first to demonstrate specific binding of human tissue prokallikrein to HK. This finding is based on several experimental approaches. First, only the high-molecular-weight form of purified native kininogens recognized biotin-prokallikrein by dot blot and direct binding assays, whereas we could not detect interactions between LK and the biotinylated kallikrein precursor. Since the heavy chains of both, LK and HK, are identical [7], this result maps the domain important for prokallikrein interaction to the light chain of HK. Structural features of the zymogen are required for complex formation with HK because mature kallikrein displayed low affinity for kininogen indistinguishable from background binding. Second, ligand blotting with denatured human plasma proteins and unmodified zymogen identified a major prokallikrein-binding protein corresponding to HK. This biochemical evidence was supported by the observation that plasma from individuals deficient in HK was also deficient in prokallikrein binding. Since native as well as denatured HK possessed strong affinity for the kallikrein precursor, we conclude that tissue prokallikrein and HK interact as native proteins.

The in vitro binding of recombinant tissue prokallikrein to purified HK is characterized by one saturable site with an apparent dissociation constant of 690 nM, which appears relatively moderate compared to a 50-fold higher HK affinity for plasma prokallikrein [16]. However, previous studies to identify endothelial proteins which bind to HK demonstrated only weak interactions between recombinant HK-binding protein p33 and its ligand but high affinity towards cell-bound p33 [17]. Conceivably, immobilization of HK on cell surfaces alters its ability to interact with potential ligands by conformational changes and/or putative modulator proteins.

One implication of the finding that HK is a prokallikrein-

binding protein relates to previous reports on neutrophil docking sites for the zymogen of tissue kallikrein [9,10]. Although we have no direct evidence demonstrating HK as a neutrophil-bound prokallikrein receptor, HK has been shown on the surface of many cardiovascular cells such as platelets, endothelial cells, and neutrophils [7], and our results are not incompatible with the hypothesis that HK docked on cell membranes may function as an acquired receptor for tissue prokallikrein. Thus, although the participation of other cell surface proteins is not excluded, the current study implicates the high-molecular-weight form of kininogens as a protein which may contribute prominently to the binding function of neutrophils towards the tissue kallikrein precursor. Definite evidence to clarify this intriguing suggestion must await additional experiments to identify the exact nature of the neutrophil-binding site for tissue prokallikrein.

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References

[1] Clements, J.A. (1997) in: The Kinin System (Farmer, S.G., Ed.), pp. 71–97, Academic Press, San Diego.
[2] Selwyn, B.M., Figueroa, C.D., Fink, E., Swan, A., Dieppe, P.A. and Bhoola, K.D. (1989) Ann. Rheum. Dis. 48, 128–133.
[3] Proud, D. and Vio, C.P. (1993) Am. J. Respir. Cell Mol. Biol. 8, 16–19.
[4] Clements, J.A. (1989) Endocr. Rev. 10, 393–419.
[5] Naidoo, Y., Snyman, C., Raidoo, D.M., Bhoola, K.D., Kemme, M. and Müller-Esterl, W. (1999) Br. J. Haematol. 105, 599–612.
[6] Angermann, A., Rahn, H.-P., Hektor, T., Fertig, G. and Kemme, M. (1992) Eur. J. Biochem. 206, 225–233.
[7] Bhoola, K.D., Figueroa, C.D. and Worthy, K. (1992) Pharmacol. Rev. 44, 1–80.
[8] Wu, H.F., Venezie, R.D., Cohen, W.M., Jenzano, J.W., Featherstone, G.L. and Lundblad, R.L. (1993) Agents Actions 38, 27–31.
[9] Kemme, M., Podlich, D., Raidoo, D.M., Snyman, C., Naidoo, S. and Bhoola, K.D. (1999) Biol. Chem. 380, 1321–1328.
[10] Anders, J. and Kemme, M. (1994) FEBS Lett. 348, 166–168.
[11] Figueroa, C.D., Henderson, L.M., Kaufman, J., De La Cadena, R., Colman, R.W., Müller-Esterl, W. and Bhoola, K.D. (1992) Blood 79, 754–759.
[12] Wachtfogel, Y.T., DeLa Cadena, R., Kunapuli, S.P., Rick, L., Miller, M., Schultze, R.L., Altieri, D.C., Edgington, T.S. and Colman, R.W. (1994) J. Biol. Chem. 269, 19307–19312.
[13] Henderson, L.M., Figueroa, C.D., Müller-Esterl, W. and Bhoola, K.D. (1994) Blood 84, 474–482.
[14] Motta, G., Rojkjaer, R., Hasan, A.A.K., Cines, D.B. and Schmaier, A.H. (1998) Blood 91, 516–528.
[15] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672.
[16] Renné, T., Dedio, J., Meijers, J.C.M., Chung, D. and Müller-Esterl, W. (1999) J. Biol. Chem. 274, 25777–25784.
[17] Herwald, H., Dedio, J., Kellner, R., Loos, M. and Müller-Esterl, W. (1996) J. Biol. Chem. 271, 13040–13047.