Isolation and Characterization of the Kininogen-binding Protein p33 from Endothelial Cells

IDENTITY WITH THE gC1q RECEPTOR*

(Received for publication, February 26, 1996, and in revised form, March 14, 1996)

Heiko Herwald‡§, Jürgen Dediots, Roland Kelner§, Michael Loos, and Werner Müller-Esterl§**

From the Institute for Physiological Chemistry and Pathobiology and the Institute of Medical Microbiology and Hygiene, Johannes Gutenberg University at Mainz, D-55099 Mainz, Federal Republic of Germany

Kinogens, the precursor proteins of the vasoactive kinins, bind specifically, reversibly, and saturably to platelets, neutrophils, and endothelial cells. Two domains of the kininogens expose major cell binding sites: domain D3 that is shared by H- and L-kininogen and domain D5i, that is exclusively present in H-kininogen. Previously we have mapped the kininogen cell binding sites to 27 residues of D3 ("LDCC27") and 20 residues of D5i ("HKH20"), respectively (Herwald, H., Hasan, A. A. K., Godovac-Zimmermann, J., Schmaier, A. H., and Müller-Esterl, W. (1995) J. Biol. Chem. 270, 14634–14642; Hasan, A. A. K., Cines, D. B., Herwald, H., Schmaier, A. H., and Müller-Esterl, W. (1995) J. Biol. Chem. 270, 19256–19261). The corresponding kininogen acceptor site(s) exposed by the cell surfaces are still poorly defined. Using a non-ionic detergent, Nonidet P-40, we have been able to solubilize kininogen binding sites from an endothelial cell line, EA.hy926, in their functionally active form. Affinity chromatography of the solubilized kininogen binding sites on HKH20, a synthetic peptide representing the D5i cell binding site, allowed us to isolate a 33-kDa protein ("p33") that binds specifically and reversibly to H-kininogen with a KD of 2 nM. Preparative SDS electrophoresis followed by NH2-terminal amino acid sequence analysis identified the kininogen-binding protein p33 as the gC1q receptor, an extrinsic membrane protein that interacts with the globular domains of the complement component C1q. The purified p33 binds C1q with moderate affinity, KD = 240 ± 10 nM. Recombinant expression of the corresponding cDNA in Escherichia coli demonstrated that p33 binds H-kininogen, but not L-kininogen. Peptide HKH20 but not peptide LDC27 inhibited binding of H-kininogen to the recombinant p33 in a concentration-dependent manner, indicating that H-kininogen binds to p33 via domain D5i. Recombinant p33 efficiently inhibited the binding of H-kininogen to EA.hy926 cells. Factor XII, but not prekallikrein, competed with H-kininogen binding to p33. These findings suggest that an endothelial binding protein mediates the assembly of critical components of the kinin-generating pathway on the surface of endothelial cells, thereby linking the early events of kinin formation and complement activation.

The kinin-generating pathway has been implicated in various physiological and pathophysiological processes, i.e. hypertension, edema formation, inflammation, and pain (1). The large precursor proteins of the kinins, i.e. high (H-) and low (L-) molecular weight kininogen, are synthesized in the liver and secreted into the plasma (2). Following their release from the kininogens by the action of kallikreins, the effector peptides, kinins, are rapidly degraded with a half-life of ~15 s in the plasma (3). Hence the kinins are considered to be locally acting hormones; however, the molecular mechanisms underlying their regional release have remained elusive. Previous studies have demonstrated that cardiovascular cells such as platelets (4, 5), neutrophils (6), and endothelial cells (7–10) expose on their surface binding sites to which the kininogens bind in a specific, saturable, and reversible manner (11). The identity of the kininogen binding site(s) is still unknown; in the absence of biological signal(s) induced by the binding of their ligand, kininogen, their nature as "receptors" remains to be demonstrated. Binding studies have revealed that the copy number of kininogen binding sites varies extensively among the various cell types, i.e. from 103 on platelets to 106 to 107 on endothelial cells (4, 5, 7–10). At least two populations of kininogen binding sites seem to be present on cells, i.e. one site to which H-kininogen binds via its unique light chain (12) and another site which accepts both H- and L-kininogen via their common heavy chain (13). It is presently unclear whether these sites correspond to two classes of proteins or whether they reflect distinct binding properties of a single protein.

Recent studies have demonstrated that the number of kininogen binding sites on endothelial cells may be up-regulated by kinins via a protein kinase C-dependent mechanism (14). Furthermore the rate of bradykinin release from H-kininogen is modified by the presence of cell membranes (15). These findings have prompted the notion that the uptake of the circulating kininogens from the plasma by cells and the
subsequent liberation of kinins on their surfaces may represent a general mechanism by which systemically available prohormones are recruited by their target cells followed by the circumstantial release of their cognate hormones (12). By virtue of this mechanism short-lived peptide hormones such as kinins are locally released at or close to their site of action.

Previously we have demonstrated that ^125I-labeled H-kininogen binds to platelets and endothelial cells via domain D3 (16). Using an antibody-directed strategy we have precisely mapped the cell binding site of the kininogen heavy chain to a segment of 27 amino acid residues ("LDC27") located in the carboxy-terminal portion of D3 (13). Screening a peptide library we have localized the cell binding site of the H-kininogen light chain to a segment of 20 residues ("HKH20") present in the histidine-rich region of domain D5H (12). The corresponding peptide, HKH20, bound specifically, reversibly, and saturably to endothelial cells with an apparent dissociation constant of 230 nM. In line with these findings the intermediate domain D4, which separates D3 and D5H in H-kininogen, contributes to the optimum binding of kininogen to endothelial cells (17). Mutual displacement of H- and L-kininogen from the endothelial cell surface indicates that the two proteins which share their NH2-terminal domains, D1 through D4, use complementary binding sites for the kininogens (15).

Here we set out to isolate, identify, and characterize a kininogen-binding protein from an endothelial cell line EA.hy926. Applying affinity chromatography on immobilized peptide HKH20 we have purified a cellular protein of 33 kDa ("p33") to apparent homogeneity. NH2-terminal sequence analysis identified the endothelial p33 as a membrane protein previously recognized as the receptor for the globular heads of H-kininogen (18). Peptides were synthesized in their amide form on a 9050 Pep-Synthesizer (Milligen, Eschborn, Germany) using Fmoc-(9-fluorenyl)-methoxycarbonyl)/HOBt chemistry and a Fmoc-amide polyethylene glycol polystyrene resin (Milligen). After cleavage from the resin the peptides were purified by reversed-phase HPLC and characterized by sequence analysis and/or mass spectrometry. The sequence data of the peptides used in this study are compiled in Table I. Antisera to synthetic peptides were raised in rabbit using standard immunization procedures (20).

Cell Culture—EA.hy926 cells (21) were cultured under standard conditions in Dulbecco’s modified Eagle’s medium with high glucose (4.5 g/liter), supplemented with 10% (v/v) fetal bovine serum, 100 μM hypoxanthine, 0.4 μM amnion, 16 μM thymidine (HAT), and antibiotics. Human umbilical vein endothelial cells (HUVECs) were prepared according to the method of Jaffe (22).

Biotinylation of H-kininogen and Peptides—Lysophosphatidylcholine (100 μg) was dissolved in 0.1 ml NaHCO3, pH 8.0, containing 10 μg of biotin-e-aminoacaproyl-N-hydroxysuccinimide (biotin-X-NHS). After incubation for 4 h at 4°C, the mixture was concentrated using a Microcon-10 column (Amicon, Beverly, MA) with a 10-kDa cutoff. Peptides HKH20 and LLT27 (1 mg each) were dissolved in 0.1M NaHCO3, pH 8.0, containing 100 μg of biotin-X-NHS. After incubation for 4 h at 4°C the mixtures were separated by HPLC. Biotinylation of the proteins and peptides was monitored by an indirect enzyme-linked immunosorbent assay (ELISA). Serial dilutions (2^n) of biotinylated H-kininogen, biotinyl-HKH20, or biotinyl-LLT27 were coated onto a microtiter plate for 1 h at 37°C. Nonspecific binding was determined by the subtraction of biotinylated peptide HKH20, respectively. The cells were washed five times with HEPES-Tyrode’s buffer or with biotinylated HKH20 (starting concentration 100 nM) or with unlabeled peptide HKH20, respectively. The cells were washed five times with HEPES-Tyrode’s buffer or with biotinylated HKH20 (starting concentration 100 nM) or with unlabeled peptide HKH20, respectively. The cells were washed five times with HEPES-Tyrode’s buffer. Cell-bound probes were detected by the preformed biotin-avidin-peroxidase complex (2 μg/ml) and the substrate solution, ABTS/H2O2 for 30 min. The change of absorbance was read at 405 nm.

Binding of Biotinylated H-kininogen and Biotinylated HKH20 to EA.hy926 Cells—The binding assay was done as described (13) with minor modifications. Briefly, EA.hy926 cells grown to confluence on 96-well microtiter plates (6 × 10^4 cells/well) were washed five times in 0.135 mM NaCl, 2.7 mM KCl, 11.9 mM NaHCO3; 0.36 mM Na2HPO4, 147 mM HEPES, pH 7.35 (HEPES-Tyrode’s buffer) containing 3.5 mg/ml dextrose and 50 mM ZnCl2. The cells were incubated with serial dilutions (2^n) of biotinylated H-kininogen (starting concentration 100 nM in HEPES-Tyrode’s buffer) or with biotinylated HKH20 (starting concentration 1.2 μM) for 1 h at 37°C. Nonspecific binding was determined by the preformed biotin-avidin-peroxidase complex (2 μg/ml) and the substrate solution, ABTS/H2O2 for 30 min. The change of absorbance was read at 405 nm. For competitive cell binding studies, the cells were incubated with serial dilutions (2^n) of biotinylated H-kininogen (starting concentration 100 nM) or with unlabeled recombinant MBP-p33 fusion protein or unfused MBP as the controls.

**Table I**

| Peptide | Sequence | Position | Comments |
|---------|----------|----------|----------|
| LDC27   | LDCNAEVYYPWKKYIYTPVNCQGGM | 331–357 | H/L-kininogen, D3 |
| VSP21   | VSFPHTSAMFADQEDRSGKE | 384–404 | H-kininogen, D5H |
| GKL19   | GKQGHTBRHDGHEKRKR | 402–420 | H-kininogen, D5H |
| HNL21   | HNLGGHHEKQDDTHGHRQ | 421–441 | H-kininogen, D5H |
| GLG19   | GLGGHEQQIGGKLHGF | 442–460 | H-kininogen, D5H |
| FKL20   | FKDLLDIEGQVLHDLVDGI | 459–478 | H-kininogen, D5H |
| HKH20   | HKGGHHEGGQKKGKKHGKH | 479–498 | H-kininogen, D5H |
| KHG20   | KHGNKXHHGKGGKKGKH | scrambled HKH20 |
| CHIT28  | CHTGGFADSLFDEIEKEEKIQKHD | 75–101 | p33/gC1qR |
| KND29   | KNDGKXALVLCHYDPEQVQDEAED | 174–202 | p33/gC1qR |
| CDR31   | CDRGVDNFADELVELSTALEAGFTEFLD | 245–274 | p33/gC1qR |
| LLT27   | LLTSSRTLPFHSKXITYPGGCGEL | Unrelated |

---

**ExPERIMENTAL PROCEDURES**

Sources of Proteins and Peptides—H-kininogen was isolated from human plasma (18) with modifications described previously (17). The complement component C1q was purified from outdated human plasma (19). Peptides were synthesized in their amide form on a 9050 Pep-Synthesizer (Milligen, Eschborn, Germany) using Fmoc-(N-(9-fluorenyl)-methoxycarbonyl)/HOBt chemistry and a Fmoc-amide polyethylene glycol polystyrene resin (Milligen). After cleavage from the resin the peptides were purified by reversed-phase HPLC and characterized by sequence analysis and/or mass spectrometry. The sequence data of the peptides used in this study are compiled in Table I. Antisera to synthetic peptides were raised in rabbit using standard immunization procedures (20).
Binding of 125I-Labeled H-kininogen to EA.hy926 Cells—EA.hy926 cells were grown to confluence in a 96-well tissue culture plate, coated to 4°C for 30 min, and washed three times with HEPES-Tyrode's buffer. The cells were incubated with decreasing concentrations (20 μM, 10 μM, 2 μM, 200 μM, 20 μM, and 2 μM) of 125I-H-kininogen for 60 min at 4°C to achieve equilibrium. The cells were washed again, removed from the plate, solubilized in the same buffer including 1% (v/v) Nonidet P-40, 4 mM CHAPS, or 10 μg/ml benzamidine hydrochloride, 10 μg/ml phenylmethylsulfonyl fluoride. The detached cells were homogenized using an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) twice for 30 s each. After centrifugation at 400 × g for 10 min at 4°C, the supernatant was collected and centrifuged at 30,000 × g for 20 min at 4°C. The resultant pellet was washed three times in HEPES-Tyrode's buffer containing 10 μg/ml benzamidine hydrochloride and 10 μg/ml phenylmethylsulfonyl fluoride (buffer A). Solubilization of the membrane pellet was done in the same buffer including 1% (v/v) Nonidet P-40, 4 mM CHAPS, or 10 μg/ml benzamidine hydrochloride, 10 μg/ml phenylmethylsulfonyl fluoride (buffer B). The bound proteins were eluted with 10 μM of 1,25-m and centrifuged at 4°C. Unbound polypeptides were removed by washing the gel with 100 μl of buffer B. Fresh gel was added, incubating the gel with 10 μl of 1.0 μM ethanediamine for 2 h at room temperature followed by washing with 100 μl of HEPES-Tyrode's buffer.

Isolation of Kininogen-binding Proteins from EA.hy926 Cells—Two methods were used to isolate kininogen-binding proteins. A: 10 ml of the membrane solubilize of EA.hy926 cells in HEPES-Tyrode's buffer containing 1% (v/v) Nonidet P-40, 4 mM CHAPS, 0.5% (w/v) desoxycholate for 60 min at 4°C. The solubilize was centrifuged at 20,000 × g for 30 min at 4°C and the clear supernatant removed and stored at −80°C until use.

Preparation of the Affinity Gels—To affinity-purify the kininogen-binding proteins from the EA.hy926 cell solubilizates, peptide HKH20 and H-kininogen were covalently coupled to Affi-Gel 10 (Bio-Rad, Munich, Germany) following the manufacturer's instructions. The gel (2.0 ml bed volume) was washed with 10 ml of H2O at 4°C, 10 ml (2 mM) of a 1 mg/ml solution of HKH20 (H-kininogen) in 0.1 M NaHCO3, pH 8.0, was added, and the resultant suspension was incubated overnight at 4°C. Unbound polypeptides were removed by washing the gel with 100 μl of buffer B. Fresh gel was added, incubating the gel with 10 μl of 1.0 mM ethanediamine for 2 h at room temperature followed by washing with 100 μl of HEPES-Tyrode's buffer.

RNA Isolation—For total cellular RNA isolation, 1–2 × 106 HUVECs were washed in ice-cold PBS and lysed in 4 ml guanidinium isothiocyanate, 0.5% (w/v) sarcosyl, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, and extracted by phenol/chloroform as described (30).

Preparation of the Affinity Gels—Oligonucleotide synthesis—Oligonucleotides were synthesized by Rothe (Karlsruhe, Germany). The S′-primer (5′-CGGATTCCTGGA-CACCGACGGAGAAGAAG-3′) containing a flanking EcoRI restriction site corresponds to the first codon (nucleotide residue 220–222) of the nucleotide sequence encoding the mature cG1q receptor, accession number X75913) (33). The S′-primer (5′-TGTTCTAGACTGCTC- TGCAAATCT-3′) corresponds to the nucleotide sequence comprising the translational stop codon of the cG1q receptor cDNA (nucleotide residue 1587–1591) flanked by 5′ and 3′ noncoding regions.

Reverse Transcriptase Polymerase Chain Reaction—The DNA synthesis was performed in a total volume of 20 μl containing 1 μg of total cellular RNA, 200 units of Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Schwalbach, Germany), 1 μM dNTPs, 10 units of RNAsin (Boehringer, Mannheim, Germany), reverse transcriptase buffer, and 100 ng of oligo(dt)18 (Rothe). Reverse transcription was performed at 37°C for 2 h. The reaction volume was adjusted to 1 ml with 10 μM Tris-HCl, pH 8.0, 1 mM EDTA. Five μl of the reverse transcription reaction mixture was added to 95 μl of a polymerase mixture that contained 2 units of Taq polymerase, 25 pmol each of the 5′- and 3′-primers (see above), 250 μM dNTPs, 10 mM Tris-HCl, 1.5 mM MgCl2, 50 μg/ml BSA, 15 μg/ml of prekallikrein, incubated (1 h, 37°C) with serial dilutions (2n) of H-kininogen, P33(5′-cG3GAATTCCTGGA-CACCGACGGAGAAGAAG-3′) containing a flanking EcoRI restriction site corresponds to the first codon (nucleotide residue 220–222) of the nucleotide sequence encoding the mature cG1q receptor, accession number X75913) (33). The S′-primer (5′-TGTTCTAGACTGCTC- TGCAAATCT-3′) corresponds to the nucleotide sequence comprising the translational stop codon of the cG1q receptor cDNA (nucleotide residue 1587–1591) flanked by 5′ and 3′ noncoding regions.

RNA was determined in a 96-well plate with 0.5% (w/v) NaOH, and the cell-associated radioactivity counted in a counter (Packard, Downers Grove, IL). The cell-associated radioactivity was determined in a 96-well plate with 0.5% (w/v) NaOH, and the cell-associated radioactivity counted in a counter (Packard, Downers Grove, IL). The cell-associated radioactivity was determined in a 96-well plate with 0.5% (w/v) NaOH, and the cell-associated radioactivity counted in a counter (Packard, Downers Grove, IL).
RESULTS

Binding of Biotinylated H-kininogen to EA.hy926 Cells—We have demonstrated previously that biotinylated H-kininogen and a peptide representing its cellular docking site on domain D5H, biotinylated HKH20, bind to endothelial cells from human umbilical vein (12). To investigate whether the same probes would also bind to a permanent cell line, EA.hy926, derived from HUVECs (21), we performed a binding study (Fig. 1). A dose-dependent binding of biotinylated H-kininogen to EA.hy926 cells was observed at concentrations of ≥10 nM. Incubation in the presence of a 6000-fold molar excess of unlabeled peptide HKH20 derived from the kininogen domain D5H (12) significantly reduced the binding of biotinylated H-kininogen (Fig. IA). The failure of the peptide HKH20 to completely block the binding of biotinylated H-kininogen reflects the fact that the H-kininogen binding to the endothelial cell is in part mediated by domain D3 (13). Direct binding of biotinylated peptide HKH20 was observed at concentrations of ≥100 nM; this binding was almost completely inhibited in the presence of a 1000-fold molar excess of the unlabeled peptide (Fig. IB). The specificity of the test system was further demonstrated by unrelated probes, biotinylated peroxidase, or biotinylated peptide LLT27, which failed to show specific binding to the EA.hy926 cells. We conclude that the endothelial cell line, EA.hy926, exposes specific kininogen binding sites.

Characterization of H-kininogen Binding Sites on EA.hy926 Cells—To determine the affinity and the number of the binding sites, we employed 125I-labeled H-kininogen over a large concentration range (16). The specific binding of H-kininogen was calculated as the difference of 125I-H-kininogen binding in the absence (total binding) or presence of a 1000-fold molar excess of unlabeled peptide HKH20 (unspecific binding). The data analysis by the LIGAND algorithm (26) revealed for H-kininogen an apparent dissociation constant (K_D) of 10 ± 4 nM for EA.hy926 cells and a total number of binding sites (B_max) of 3.8 ± 0.3 × 10^5/cell.

Isolation of Kininogen Binding Sites from EA.hy926 Cells—To isolate kininogen binding sites, we prepared membranes from EA.hy926 cells by hypotonic shock and tested by surface plasmon resonance spectroscopy (BIAlite) the efficiency of the various detergents to solubilize intact binding sites. Incubation in the presence of a 6000-fold molar excess of unlabeled peptide HKH20 (unspecific binding). The data analysis by the LIGAND algorithm (26) revealed for H-kininogen an apparent dissociation constant (K_D) of 10 ± 4 nM for EA.hy926 cells and a total number of binding sites (B_max) of 3.8 ± 0.3 × 10^5/cell.

Isolation of Kininogen Binding Sites from EA.hy926 Cells—To isolate kininogen binding sites, we prepared membranes from EA.hy926 cells by hypotonic shock and tested by surface plasmon resonance spectroscopy (BIAlite) the efficiency of the various detergents to solubilize intact binding sites. Incubation in the presence of a 6000-fold molar excess of unlabeled peptide HKH20 (unspecific binding). The data analysis by the LIGAND algorithm (26) revealed for H-kininogen an apparent dissociation constant (K_D) of 10 ± 4 nM for EA.hy926 cells and a total number of binding sites (B_max) of 3.8 ± 0.3 × 10^5/cell.
association and dissociation rates, respectively, an apparent $K_D$ of 9 $\pm$ 2 nM was calculated. This $K_D$ is in good agreement with that for H-kininogen binding to the intact EA.hy926 cells (10 $\pm$ 4 nM). Hence the isolated 33-kDa protein shows the properties of a cellular kininogen-binding protein.

NH$_2$-terminal Sequence Analysis of p33—Endothelial p33 isolated by the latter purification scheme (“B”) was subjected to preparative SDS-PAGE, electroblotted onto polyvinylidene difluoride membranes, and subjected to sequence analysis. The initial yield of the Edman degradation revealed an amount of 22 pmol of p33. The starting material contained approximately 260 pmol of H-kininogen-binding protein as calculated from 3.8 $\times$ 10$^5$ binding sites/cell and a total cell number of 4.3 $\times$ 10$^8$ present on 30 dishes. Hence the yield of the isolation procedure was $\approx$8% (note that we used intact cells for the binding assay, i.e. any p33 present within the cell is unavailable for the ligand). The NH$_2$-terminal 15 amino acid residues of p33 were unambiguously determined by automated Edman degradation (Table I). A search of the protein data bases revealed a complete identity of this sequence with the amino-terminal portion of the human C1q receptor, gC1qR (31). This latter protein has been reported to have a molecular mass of 33,000 Da and to be abundant in B cells, platelets, neutrophils, and mast cells (32-34). We tentatively conclude that endothelial p33 is identical to gC1qR. To test this hypothesis we performed binding studies with purified C1q (70-100 $\mu$g/ml) on p33 covalently attached to a BiAlite sensor chip. An apparent $K_D$ of 240 $\pm$ 10 nM was calculated for the binding of C1q to p33. Together these findings suggest that endothelial p33 and gC1qR are identical proteins.

Production of Recombinant gC1qR—To further address the identity of p33 with gC1qR, we tested the affinity of recombinant gC1qR protein for H-kininogen. We deno the cDNA encoding the mature form of gC1qR from HUVECs by reverse transcriptase-polymerase chain reaction using primers derived from the known sequence of gC1qR (note that the immature form of gC1qR has an extension of 73 residues preceding the identified NH$_2$ terminus; Ref. 31). To increase the solubility of gC1qR, we expressed it as a fusion protein with MBP in E. coli (Fig. 4, lane 1). The MBP-gC1qR fusion protein was found to be primarily associated with the cytoplasmic fraction of the bacteria (Fig. 4, lane 2). To release gC1qR from the affinity-purified fusion protein (Fig. 4, lane 3), MBP-gC1qR was cleaved by factor Xa for 10 h at 37°C, and the MBP and gC1qR portions were separated by reversed-phase HPLC (Fig. 4, lanes 4 and 5). The NH$_2$-terminal sequence of the recombinant gC1qR, Leu-His-Thr-Asp-Gly, was identical to that of the native receptor (31) and of p33, respectively (Table I).

Kininogen Binding Properties of Recombinant gC1qR—To test for the binding properties of the purified fusion protein, an indirect ELISA was employed. Microtiter plates were coated with increasing concentrations of H-kininogen, C1q, or L-kininogen, followed by MBP-gC1qR. Bound protein was detected by a polyclonal antibody directed to the MBP portion of the fusion protein (Fig. 5A). H-kininogen bound MBP-gC1qR most efficiently whereas the affinity of C1q was significantly lower. No specific binding was observed with L-kininogen demonstrating that the gC1qR binding site is comprised by the unique light chain of H-kininogen. Application of MBP alone did not result in a significant binding of H- or L-kininogen. However a minor though significant binding of MBP to C1q was found (Fig. 5B), suggesting that the MBP portion contributes to the binding of the MBP-gC1qR fusion protein (Fig. 5A). The binding constant of H-kininogen for MBP-gC1qR ($K_D = 15 \pm 5$ nM) was similar to those observed for purified p33 (9 nM) or for native EA.hy926 cells (10 nM). Because the recombinant gC1qR exhibits the functional features of a kininogen-binding protein and the endothelial p33 has C1q binding properties, we conclude that the two entities are identical. We noted however that the recombinant protein binds H-kininogen independent of Zn$^{2+}$ (data not shown), whereas the association of kininogen to native endothelial cells is strictly Zn$^{2+}$-dependent (7). At present we cannot explain this discrepancy.

Recombinant gC1qR Competes for Kininogen Binding to EA.hy926 Cells—To further probe the functional identity of gC1qR with p33, we tested the effect of increasing concentrations of MBP-gC1qR on the cell binding of biotinylated H-

---

**Table I**

NH$_2$-terminal sequence analysis of the kininogen-binding protein p33 from endothelial cells

The results of the Edman degradation are shown as the stepwise yields in pmol (numbers in body of table) of the phenylthiohydantoin derivatives of the corresponding amino acids.

| Leu | His | Thr | Asp | Gly | Asp | Lys | Ala | Phe | Val | Asp | Phe | Leu | Ser | Asp |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 12.1| 2.1 | 4.3 | 9.6 | 5.8 | 5.1 | 2.1 | 4.8 | 5.2 | 4.5 | 3.9 | 5.9 | 4.3 | 3.0 | 4.0 |
kininogen or biotinylated peptide HKH20. Using the same experimental set-up as in Fig. 1, we found a dose-dependent effect of MBP-gC1qR on the binding of biotinylated H-kininogen (Fig. 5C) and biotinylated HKH20 (Fig. 5D) to EA.hy926 cells. We were unable to find such an effect with MBP alone or with unrelated probes, biotinylated peroxidase, or biotinylated LLT27. Together these data indicate that gC1qR has the structural and functional properties of a H-kininogen-binding protein. Hereafter we use p33 and gC1qR synonymously.

Identification of the p33 Binding Site in H-kininogen—Our previous mapping studies have demonstrated that domain D5H harbors the major cellular docking site of the H-kininogen light chain (12). To test whether the same site is involved in the binding to p33, we applied in a competitive ELISA a set of peptides covering the entire domain D5H (Table I). Microtiter plates coated with H-kininogen were incubated with recombinant p33 (fused to MBP) in the presence of increasing concentrations of peptides GKE19, HKH20, and HNL21. Peptide HKH20 was by far the most potent inhibitor of p33 binding with an apparent IC50 (concentration at 50% inhibition) of 440 nM (Fig. 6A). Peptide GKE19 was less effective (IC50 > 500 μM), and HNL21 had no effect on the complex formation. Likewise peptides VSP21, GLG19, and FKL20 covering other portions of D5H were ineffective (data not shown). For control we used peptide KHG20, a peptide of identical amino acid composition but distinct ("scrambled") sequence as HKH20. Peptide KHG20 showed no inhibitory effect (data not shown), indicating that the high affinity of peptide HKH20 for p33 is sequence-specific and not solely due to an accumulation of positively charged side chains (see Table I). These results are in good agreement with the data obtained from intact endothelial cells where HKH20 was by far the most efficient inhibitor whereas GKE19 and HNL21 were by a factor of 103 less effective (12). We also tested peptide LDC27, which covers the major endothelial cell binding site of the kininogen heavy chain (13). This peptide was not able to inhibit binding of H-kininogen to p33 (data not shown).

We conclude that the major contact site of H-kininogen for p33 is located at the extreme carboxyl-terminal end of D5H.

In an initial attempt to localize the corresponding kininogen binding site of p33, we synthesized three peptides, CHT28, KND29, and CDR31, derived from the NH2 terminus, the center portion and the COOH terminus of p33 (Table I). None of these peptides interfered with the complex formation (data not shown), indicating that the H-kininogen binding activity is not associated with these linear sequences.

Binding of a Kininogen-Prekallikrein Complex to p33—H-kininogen circulates in plasma as bimolecular complexes with the contact phase factors prekallikrein or factor XI (35). Because the interaction between the zymogens and the kininogen is mediated by its domain D6H (36, 37), the question arose whether the adjacent domain D5H would be available for docking of these complexes to p33. To exemplify this problem, we immobilized human plasma prekallikrein on a microtiter plate and added increasing concentrations of H-kininogen followed...
The hypothetical scheme depicts the local assembly of prekallikrein (PK), factor XI (FXI), H-kininogen (HK), and p33 on the surface of endothelial cells. Indirect evidence suggests that factor XII (FXII) can bind to the same docking structure via p33. The D3 binding site of endothelial cells, which is distinct from p33, and the p33-associated proteins of 60 and 100 kDa (p60/p100) are marked by a solid box; their precise nature (transmembrane versus extrinsic) is unknown.

**FIG. 6.** Interaction sites between H-kininogen and p33. A, microtiter plates coated with 2.5 μg/ml H-kininogen were incubated with 2.5 μg/ml of MBP-p33 in the presence of increasing concentrations of peptides HKH20 (○), GKE19 (○), or HNL21 (■) for 1 h at 37°C. The bound MBP-p33 was detected by antibodies to MBP followed by a peroxidase-labeled secondary antibody. The results for peptides VSP21, GLG19, FKl20, LDC27, CHT28, KND29, and CDR31 were indistinguishable from those of peptide HNL21 (data not presented). B, sandwich ELISA using 5 μg/ml of prekallikrein (□) as the coating agent. Serial dilutions (2⁻) of H-kininogen were applied, followed by the MBP-p33 fusion protein and the indirect peroxidase detection system. For control, 1 μg/ml of a polyclonal antibody to the H-kininogen heavy chain (○) or 5 μg/ml of α₂HS glycoprotein (○) were used. C, the indirect ELISA was performed as detailed for A except that serial dilutions (2⁻) of factor XII (○), C1q (○), or α₂HS glycoprotein (□) were used as the competitors. ○, prekallikrein; □, peptide competitors; ■, protein competitors; other icons are the same as those in Fig. 1.

by the MBP-p33 and an antibody to the MBP fusion part. For a positive control, we coated the plate with an antibody to the kininogen heavy chain, which leaves the light chain domains accessible; for a negative control, we coated the plate with an unrelated human plasma protein, α₂HS glycoprotein. A significant binding of MBP-p33 to the preformed H-kininogen-prekallikrein complex was found (Fig. 6B). MBP-p33 bound also to the antibody-kininogen complex but not to α₂HS glycoprotein; MBP alone was without effect (not shown). The capacity of p33 to bind to the preformed H-kininogen-prekallikrein complex opens the possibility that prekallikrein anchors indirectly to the surface of endothelial cells via H-kininogen.

**FIG. 7.** Assembly of the components of kinin-forming pathway on endothelial cells. The hypothetical scheme depicts the local assembly of prekallikrein (PK), factor XI (FXI), H-kininogen (HK), and p33 on the surface of endothelial cells. Indirect evidence suggests that factor XII (FXII) can bind to the same docking structure via p33. The D3 binding site of endothelial cells, which is distinct from p33, and the p33-associated proteins of 60 and 100 kDa (p60/p100) are marked by a solid box; their precise nature (transmembrane versus extrinsic) is unknown.

**DISCUSSION**

Homeostasis of the body is maintained by intercellular communication via autocrine, paracrine, and exocrine transmitters and hormones. Two principal pathways may govern the actions of exocrine mode. One such pathway comprises the biosynthesis of the precursor hormones in specific tissues, their proteolytic processing to the cognate peptide hormones in or next to their producing cells, the stimulus-regulated release of the stored peptide hormones, and the transport of the hormones to and their action on distant target cells. This pathway is well established, e.g. for the proinsulin system. The other pathway comprises the biosynthesis and constitutive secretion of precursor hormones by specific tissues, the transport and attachment of prohormones to distant cells; the stimulus-induced processing of the surface-bound prohormones at or next to their target cells, and the circumscribed release of the cognate peptide hormones at their site of action. One candidate system that meets the criteria of the latter pathway is the kallikrein-kinin system. Several lines of evidence support this notion: (i) the bulk of kininogens are synthesized by hepatocytes and secreted into the plasma (2); (ii) under physiological conditions the circulating kininogens are present in their native, kinin-containing form (2); (iii) kininogens bind to cardiovascular cells such as the endothelial cells with high affinity and specificity (4–11);
investigations have provided indirect evidence from displaced membrane and contribute to the cell surface binding of the existence of other endothelial proteins that tether p33 to secured to the membrane by docking to intrinsic membrane residues (31). It is presently unclear where the processing of the precursor form occurs and by which mechanisms and demonstrated by several independent investigators (4, 6–10); however, the nature of the kininogen binding site(s) has remained elusive. In this work we identify for the first time one such elusive. In this work we identify for the first time one such kininogen-binding protein, p33, as the receptor for C1q, gC1qR. Previous studies by Ghebrehiwet, Reid, and co-workers have demonstrated that p33/gC1qR is present on the surface of a variety of cardiovascular cells such as B cells, platelets, neutrophils, and mast cells (31–34, 38). This protein is biosynthesized in a pre-proform of 282 amino acid residues, including a predicted signal peptide of 16 residues and a propeptide (31). It is presently unclear where the processing of the precursor form occurs and by which mechanisms and routes the mature protein is transported to the surface of the producing cells. Previous studies have demonstrated that p33/gC1qR is associated with the membrane fraction of B cells (31); our preliminary results from immunoblotting studies with EA.hy926 cells using a polyclonal antibody to recombinant p33 support this finding (data not shown). At present the mode of attachment of p33 to the membrane is unknown. The apparent lack of a consensus site for glycosylphosphatidylinositol anchoring and the absence of a hydrophobic stretch sufficiently long to allow spanning of a membrane suggests that p33 represents an extrinsic membrane protein. Protein p33 could be secured to the membrane by docking to intrinsic membrane protein(s) and/or by electrostatic interactions (Fig. 7). Our finding that the affinity chromatography on H-kininogen yielded three proteins of 33, 60, and 100 kDa, respectively, might point to the existence of other endothelial proteins that tether p33 to the membrane and contribute to the cell surface binding of kininogens. Thus far we have been unable to identify the exact nature of these copurified proteins. Our present study does not address the identity of kininogen-binding proteins on cells other than endothelial cells. Previous investigations have provided indirect evidence from displacement studies with monoclonal antibodies that the leukocyte integrin Mac-1 (CD11b/18; \( \alpha_m \beta_2 \)) could be involved in the binding of H-kininogen to neutrophils (41). Since Mac-1 expression is restricted to leukocytes, the possibility remains that neutrophils may bind kininogens via distinct docking protein(s). Furthermore p33 may act in concert with other proteins such as the cell adhesion protein Mac-1 to secure the binding and processing of kininogens on cells surfaces. At present we do not know whether p33 represents solely an anchor for kininogen presentation on the endothelial surface (“acceptor”) or whether it forms part of a larger complex which transduces signals to the interior of the cell upon kininogen binding (“receptor”). The availability of peptide surrogates such as HKH20, which bind to endothelial cells with high affinity, and the development of specific antibody probes, which interfere with the binding of kininogens to the cell surface structures, will allow an in-depth analysis of this problem. We anticipate that the detailed study of cellular prohormone-binding proteins will deepen our understanding of the molecular mechanisms underlying the exocrine mode of hormone action.
Isolation and Characterization of the Kininogen-binding Protein p33 from Endothelial Cells: IDENTITY WITH THE gC1q RECEPTOR
Heiko Herwald, Jürgen Dedio, Roland Kellner, Michael Loos and Werner Müller-Esterl

J. Biol. Chem. 1996, 271:13040-13047.
doi: 10.1074/jbc.271.22.13040

Access the most updated version of this article at http://www.jbc.org/content/271/22/13040

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 26 of which can be accessed free at
http://www.jbc.org/content/271/22/13040.full.html#ref-list-1