Identification of cytokeratin 1 as a binding protein and presentation receptor for kininogens on endothelial cells

AHMED A. K. HASAN, TIMOTHY ZISMAN, AND ALVIN H. SCHMAIER*

Hematology/Oncology Division, Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109-0640

Communicated by Oscar D. Ratnoff, Case Western Reserve University, Shaker Heights, OH, January 5, 1998 (received for review February 3, 1997)

ABSTRACT A kininogen binding protein(s), a putative receptor, was identified on endothelial cells. A 54-kDa protein was isolated by a biotin–high molecular mass kininogen (HK) affinity column that, on aminoterminal sequencing of tryptic digests, was identified as cytokeratin 1. Multiple antibodies directed to cytokeratin 1 reacted with a 54-kDa band on immuno- blot of lysates of endothelial cells. On laser scanning confocal microscopy, cytokeratin 1 antigen was found on the surface of endothelial cells. Cytokeratin 1 antigen also was detected on endothelial cell membranes by flow cytometry. Moreover, an antipeptide antibody to a sequence unique to cytokeratin 1 also specifically bound to nonpermeabilized endothelial cells. Bi- otin–HK specifically bound to cytokeratin only in the presence of Zn2+, and cytokeratin blocked biotin–HK binding to endothelial cells. Further, HK and low molecular mass kininogen, but not factor XII, blocked biotin–HK binding to cytokeratin, and peptides of each cell binding region of HK on domains 3, 4, and 5 blocked biotin–HK binding to cytokeratin. gC1qR and soluble urokinase-like plasminogen activator receptor also inhibited biotin–HK binding to cytokeratin. These investigations identify a new function for cytokeratin 1 as a kininogen binding protein. Cytokeratins, members of the family of intermediate filament proteins, may represent a new class of receptors.

The kinogenins, high (HK) and low (LK) molecular mass kininogen, are multidomain proteins whose prime function is to deliver the vasoactive peptide bradykinin (BK). BK has multiple effects at the cellular level in the intravascular compartment. It is known to stimulate prostaglandin synthesis in endothelial cells (1, 2), induce superoxide formation (3), release tissue-type plasminogen activator (4, 5), stimulate NO formation and elevation of cGMP from endothelial cells (6, 7), and induce smooth muscle hyperpolarization factor (8). Although there are two BK receptors in the intravascular compartment (9, 10), little is known about how the liberation of BK is regulated. In plasma and on cell mem- branes there are multiple kininases that degrade BK once it is delivered to the membrane of HUVEC. Identification and characterization of the kininogen receptor(s) in the intravascular compartment (e.g., HUVEC, platelets, and granulocytes) is crucial to fully explain the binding of kinogenins to cells in the intravascular compartment (e.g., HUVEC, platelets, and granulocytes). HUVEC gC1qR is mostly intracellular (17, 18), and uPAR is not found on platelets. Thus, other candidate kinogenin receptors must exist. In this study, we identify human cytokeratin 1 as a kininogen binding protein on the membrane of HUVEC.

EXPERIMENTAL PROCEDURES

Proteins and Reagents. HK and LK were purified and characterized as reported (12, 19). HK was biotinylated as reported (11, 12). Peptides LDC27 (LDCNAEYVVPEWKKKYPTVNCQPLGM) from domain 3 of the kinogenins, MKBK (MRKPGFSPFSRSG) from domain 4 of the kinogenins, HKH20 (HKHGHGKHKKNGKHKNGKH) and HVL24 (HVLDHGKHKHKGHGHKHKNGKDKK) from domain 5 of the kini- genins, and FNO15 (FNOQPQERGDNNLTR), the factor X activation peptide, were synthesized and purified in the Protein and Carbohydrate Structure Facility of the University of Michi- gan as reported (12, 20–22). gC1qR as a fusion protein with maltose binding protein was provided generously by Werner Muller–Esterl, Johannes Gutenberg University of Mainz, Mainz, Germany. Soluble uPAR was provided generously by Douglas B. Cines, University of Pennsylvania, Philadelphia. Purified human factor XII was purchased from Enzyme Research Laboratories, South Bend, IN. Human purified cytokeratin, which is a mixture of many cytokeratins, and a rabbit anti-pancytokeratin antibody were purchased from Dako. Fab fragments of this rabbit anti- pancytokeratin antibody and rabbit IgG were provided by a procedure obtained from Pierce. Peptide acetyl-RRYDQLKSDQSRKDLSELC-amide (RRY16), which is a sequence unique to human cytokeratin 1 and which spans amino acid Arg89 to Leu105 (23), was synthesized and used to produce anti-peptide antisera (Anti-RRY16) in goats at Quality Control Biochemicals, Hop- kinton, MA. mAb AE2 directed to cytokeratins 1 and 10 was purchased from ICN, mAbs C2931, C2562, C1801, C6909, C8541, C7159, and C0791 and mouse IgG were purchased from Sigma. mAb C2931 is a mixture of anti-cytokeratin clones that contains antibodies directed to cytokeratin 4, 5, 6, 8, 10, 13, and 18. mAb C2562 contains antibodies directed to cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, and 19. mAb C1801 contains antibodies directed to cytokeratins 1, 5, 6, and 8. mAb C6909 contains antibodies...
directed to cytokeratins 1, 5, 6, 7, 8, 10, 11, and 18. mAbs C8541, C7159, and C0791 are directed to cytokeratins 8, 19, and 13, respectively.

**Endothelial Cells.** Cultures of HUVEC were established as described (11). HUVEC were purchased from Clonetics (San Diego) and propagated by using medium and growth factors from Clonetics. In preparation for cell binding studies, HUVEC were grown to confluence on fibronectin-coated, 96-well microtiter plates (Nunc). In other experiments, HUVEC were grown on 19-mm HTC-SC white slides (Cell Line Associates, Newfield, NJ) for laser scanning confocal microscopy.

**Binding of Biotin–HK to HUVEC.** Confluent HUVEC on 96-well microtiter plates (4 × 10⁶ cells/well) were washed five times in Heps–Tyrode’s binding buffer prepared as reported (11). The cells were incubated with 20 nM biotin–HK in Heps–Tyrode’s buffer containing 50 μM Zn²⁺ at 37°C for 1 h to achieve equilibrium. Nonspecific binding was determined by measuring binding in the absence of Zn²⁺, which is equivalent to binding seen with 50 μM Zn²⁺ and a 50-fold molar excess of HK (12). Cell-associated biotin–HK was detected by using Immunopure streptavidin horseradish peroxidase conjugate (Pierce) and fast-acting 3,3′,5,5′-tetramethylenbenzidine dihydrochloride (TMB-Turbo) peroxidase substrate (Pierce) as reported (11, 12, 20, 21).

**Affinity Isolation of Kininogen Binding Protein.** A biotin–HK–streptavidin affinity column was prepared by using UltraLink Immobilized Streptavidin gel from Pierce. In brief, 1–3 mg of biotin–HK was coupled to 1–2 ml of gel in 0.02 M sodium phosphate and 0.5 M NaCl (pH 7.5). HUVEC from confluent dishes were washed with 0.02 M sodium phosphate and 0.5 M NaCl (pH 7.5) containing 5 mM EDTA, 0.1 mM leupeptin, 10 mM benzamidine, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonylfluoride. Endothelial cell lysates were prepared by treating the cells with 0.02 M sodium phosphate and 0.5 M NaCl (pH 7.5) containing 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.02% sodium azide. The solubilized cells were sonicated on ice, and the sonicate was centrifuged at 12,000 g in a microcentrifuge to remove any particulate material. Because one endothelial cell contains 1 × 10⁶ sites for kininogen to bind (11), a sufficient amount of lysate was added to the column to saturate all of the bound HK. In the affinity isolation of kininogen binding proteins, usually 2–3 ml of lysate containing 50 μM Zn²⁺ was applied to the column preequilibrated with lysate buffer containing 50 μM Zn²⁺. Once the lysate was applied, the column was washed with 10 column volumes of 0.02 M sodium phosphate and 0.5 M NaCl (pH 7.5) containing 50 μM Zn²⁺ until the effluent OD₂₈₀ nm was <0.05. Protein bound to the affinity column was eluted with treatment of 0.2 M glycine (pH 2.8), which was immediately adjusted to pH 7.5 by 1 M Tris. Eluted material was electrophoresed, nonreduced, and reduced with 2% β-mercaptoethanol followed by boiling on an 8% SDS/PAGE and visualized with Coomassie blue R-250.

Affinity-purified HK binding protein(s) was concentrated and desalted on a 1.0 × 50 mm HPLC C4 column. The protein then was electrophoresed by SDS/PAGE and transferred to a poly(vinylidene difluoride) membrane, and the protein band was visualized with 50 × nonfat dry milk in 0.01 M sodium phosphate and 0.15 M NaCl (pH 7.4) (24). After electrophoresis, the affinity column was eluted with treatment of 0.2 M glycine (pH 2.8), which was immediately adjusted to pH 7.5 by 1 M Tris. Eluted material was electrophoresed, nonreduced, and reduced with 2% β-mercaptoethanol followed by boiling on an 8% SDS/PAGE and visualized with Coomassie blue R-250.
The antibody bound was detected with a peroxidase-specific substrate, 4-chloro-1-naphthol substrate (Sigma).

**Flow Cytometry.** HUVEC were detached with a nonenzymatic cell dissociation buffer containing EDTA (Life Technologies, Grand Island, NY, and GIBCO/BRL) for 5–10 min at 37°C. The cells centrifuged gently at 400 $\times$ g for 5 min and washed with Hepes–Tyrode’s binding buffer. Cells ($2 \times 10^5$) then were incubated with various mAbs or goat anti-RRY16 antiserum to human cytokeratin 1 at 1/100 dilution on ice for 1 h with occasional gentle mixing. The cells were washed by centrifugation at 400 $\times$ g three times and resuspended in Hepes–Tyrode’s buffer containing 1/250 dilution of a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse or mouse anti-goat secondary antibody, respectively. After incubating an additional 1 h in the dark, the cells were washed three more times and resuspended. The fluorescence of bound FITC-labeled secondary antibody to HUVEC was monitored with an Epics-C flow cytometer (Coulter). Light scatter and fluorescence channels were set at logarithmic gain. Laser excitation was at 488 nm. Green fluorescence was observed through a 525-nm band pass filter. The relative fluorescence intensity of $\approx 15,000$ HUVEC was analyzed in each sample.

**Results**

**Purification of HK Binding Proteins.** The biotin–HK immobilized streptavidin affinity column repeatedly isolated 54- and 102-kDa proteins from HUVEC lysates (Fig. 1A). On occasion, flow cytometry of HUVEC. Suspensions of washed, unfixed, and nonpermeabilized HUVEC were incubated with mouse IgG (unshaded curves) or mAbs C2562, C7159, C2931, or C0791, each added at 1/100 dilution. The binding of these antibodies on the HUVEC membrane was detected with a secondary antibody labeled with FITC. The flow cytogram of HUVEC alone not treated with any Ig is shown in the upper left. The box to the right of the flow cytograms represents the names of the antibody clones and the cytokeratins they react to. The data presented are representative of three experiments.

**Laser Scanning Confocal Microscopy.** HUVEC grown on microscope slides were used in laser scanning confocal microscopy experiments. Nonpermeabilized HUVEC grown on microscope slides either were or were not fixed with 2% paraformaldehyde, as reported (25). After being washed with Heps–Tyrode’s buffer, the cells were incubated for 1 h at room temperature with 1 $\mu$g/ml (or a 1/100 dilution) of mAb to cytokeratin. After washing again, they were incubated with a goat anti-mouse secondary antibody conjugated with FITC. The slides were covered with a Vectashield antifading mounting medium (Vector Laboratories) and were visualized by using a confocal fluorescence microscope (Bio-Rad). Both projection–view and optical sections were restored electronically and were processed digitally. Optical scanning and digital processing of the images were performed to determine the topographic distribution of the FITC/IgG associated with HUVEC as reported (11).

**Fig. 2.** Laser scanning confocal microscopy. (A) Paraformaldehyde (2%) fixed but nonpermeabilized HUVEC grown on microscope slides were incubated with mAbs C2562, C2931, C1801, and mouse IgG. (B) Unfixed and nonpermeabilized HUVEC grown on microscope slides were incubated with mAbs C2562, C2931, C6909, and mouse IgG. The panels to this figure are photomicrographs of the laser scanning confocal microscopy. The table between the laser scanning photomicrographs lists the mAb clones and the cytokeratins they react to. The figure is a representative presentation of multiple experiments.
a fainter 33-kDa protein was seen, but an insufficient amount was procured for sequencing. If lysates were applied in the absence of zinc ion, little material bound to the column. Both the 54- and 102-kDa proteins were blocked at the N terminus and could not be sequenced directly. Tryptic digests of the 54-kDa band were prepared for N-terminal sequencing. On the first occasion, two peptide sequences, SLDLDSIAEV and LNDMEDALQOAK, were obtained and were identified as cytokeratins including cytokeratin 1. The sequence SLDLDSIAEV is found in cytokeratins 1, 6, 5, 8, and 4 and is coded by exon 5 of the gene for cytokeratin 1 (23) (Fig. 1A). The sequence LNDMEDALQOAK is found in cytokeratins 1, 2, and 4 and is coded by exon 7 of the gene for cytokeratin 1. On a second affinity isolation, peptide ELLOQYDST1 was isolated. This sequence is coded uniquely by exon 2 of cytokeratin 1 (23). These data indicated the presence of cytokeratin 1 at 54 kDa in HUVEC; cytokeratin 1 was found to be a HK binding protein.

**Immunoblot of Endothelial Cell Lysates.** Immunoblot studies were performed to determine whether the mAb to human cytokeratin 1 could detect this antigen in HUVEC lysates (Fig. 1B). mAbs C2562, C1801, and C6909, which contained clones reactive to cytokeratin 1 antigen, were able to immunoblot a 54-kDa band in HUVEC lysates, a protein similar in size to that purified from HUVEC lysates on the HK affinity column (Fig. 1 A and B). Furthermore, neither mAb C2931 nor mouse IgG was able to immunoblot any antigen in HUVEC lysates.

**Membrane Expression of Cytokeratin 1 by HUVEC.** Initial studies showed that, by using mAb AE2, which is directed to cytokeratins 1 and 10 on fixed and permeabilized HUVEC, the majority of cytokeratin antigen was present in these cells by laser scanning confocal microscopy (data not shown). Investigations next proceeded to determine whether cytokeratin 1 could be identified on HUVEC plasma membranes. Experiments showed that a rabbit anti-human Fab to pancytokeratin specifically bound to confluent HUVEC on microtiter plates (data not shown). Laser scanning confocal microscopy was used to identify the presence and distribution of cytokeratin 1 on HUVEC. Fixed but nonpermeabilized HUVEC were examined for the presence of cytokeratin antigen (Fig. 2A). mAb C2562 (which detects cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, and 19) and mAb C1801 (which detects cytokeratins 1, 5, 6, and 8) showed cytokeratin antigen on the external membranes of HUVEC. However, mAb C2931 (which detects cytokeratins 4, 5, 6, 8, 10, 13, and 18 and mouse IgG) detected no cytokeratin antigens on HUVEC membranes (Fig. 2A). Further investigations were performed to determine whether cytokeratin antigen could be detected on the membranes of unfixed and nonpermeabilized HUVEC (Fig. 2B). mAbs C2562 and C6909 (which is directed to cytokeratin 1, 5, 6, 7, 8, 10, 11, and 18) detected cytokeratin on the membranes of these cells, whereas antibody C2931 and mouse IgG did not.

More investigations were performed to confirm that cytokeratin 1 was on the external membrane of HUVEC. mAb C2562 showed a forward scatter on a flow cytogram of unfixed and nonpermeabilized HUVEC in suspension, indicative of membrane expression of cytokeratin 1 (Fig. 3). Alternatively, mAbs C2931, C7159, and C0791 (the latter of which are directed to cytokeratins 19 and 13, respectively) did not detect cytokeratin antigen on HUVEC membranes. Furthermore, mAb C8541 (which is directed to cytokeratin 8) also failed to detect antigen on suspended HUVEC (data not shown). These data, along with the laser scanning confocal microscopy studies, showed by deduction that cytokeratin 1 is the only cytokeratin expressed on HUVEC membranes.

Investigations also were performed to demonstrate the presence of cytokeratin 1 antigen on HUVEC and in a purified cytokeratin mixture. An antipeptide antiserum (anti-RRY16) was reared to a sequence (R09RYDQLKDSQRLDSEL105C) unique to cytokeratin 1 coded by exon 2 (23). This antiserum specifically bound to nonpermeabilized confluent HUVEC grown on a microtiter plate (data not shown). Flow cytometry experiments also showed that anti-RRY16 antisera recognized cytokeratin 1 on the membrane of unfixed and nonpermeabilized HUVEC in suspension (Fig. 4A). Cytokeratin 1 antigen also was present in a purified cytokeratin preparation purchased from Dako (Fig. 4B). When anti-RRY16 antisera or its preimmune serum was incubated with the purified cytokeratins, more anti-RRY16 antibody bound in a concentration-dependent fashion to the purified cytokeratin mixture than its preimmune serum, confirming the presence of cytokeratin 1 in the preparation (Fig. 4B).

**Interactions Between HK and Cytokeratin 1.** Investigations next were performed to determine whether biotin–HK bound to purified cytokeratin (Fig. 5A). Biotin–HK specifically bound to purified cytokeratin in a solid phase assay only when 50 μM Zn 2+ was present. In the absence of Zn 2+ or when BSA coated the microtiter plate wells, no specific binding of biotin–HK occurred. Accordingly, purified cytokeratin blocked biotin–HK from binding to HUVEC (Fig. 5B). Increasing concentrations of purified cytokeratin (2 μM to 3 μM) blocked biotin–HK binding with an apparent IC50 of 1 μM, indicating that the kininogen binding site for cytokeratin occupied the same region(s) on HK that it had for binding to the HUVEC membrane. These combined data indicated that HK binds to cytokeratin and that cytokeratin competes for HK binding to HUVEC.

More studies determined the domains of kininogens that interact with cytokeratin 1 (Fig. 6A). Increasing concentrations of HK and LK but not factor XII blocked biotin–HK binding to cytokeratin (Fig. 6A). HK and LK blocked biotin–HK binding to...
cytokeratins with an IC<sub>50</sub> of 40 nM and with an IC<sub>50</sub> of 200 nM, respectively. Further experiments were performed to determine the binding region(s) of HK that were involved in its interaction with cytokeratin (Fig. 6B). Peptide MKBK from domain 4, peptides HKH20 and HVL24 from domain 5, and peptide LDC27 from domain 3 blocked biotin–HK binding to cytokeratin with an IC<sub>50</sub> of 100 μM for MKBK and with an IC<sub>50</sub> of ~6 μM for the others. Peptide FNQ15, which is the factor X activation peptide, had no influence on biotin–HK binding to cytokeratin. These data indicated that HK binding to purified cytokeratin was mediated through the same binding regions on the three different domains it uses to bind to HUVEC (12, 20, 21).

Last, investigations were performed to determine whether other kininogen binding proteins influenced biotin–HK binding to cytokeratin (Fig. 7). The fusion protein of gC1qR and soluble uPAR both at 1 μM blocked biotin–HK binding to cytokeratin. Factor XII did not. These data indicated that HK interacted with cytokeratin through the common domain(s) it used to interact with the other candidate kininogen binding proteins, gC1qR and uPAR.

**DISCUSSION**

The finding that cytokeratin 1 was a kininogen binding protein, a putative receptor, on endothelial cells was not expected. Cytokeratins are the major protein constituent of skin, and, in particular, cytokeratin 1 is most commonly found in skin above the basal membrane layer in cells on their migration to desquamation. To date, it is our understanding that cytokeratin 1 has not been described to be associated with umbilical vein endothelial cells. Independent of the affinity isolation, we have confirmed that cytokeratin 1 is present on HUVEC membranes. First, multiple commercially available mAbs to cytokeratin 1 recognized it on HUVEC by direct binding experiments, immunofluorescence, and flow cytometry. Second, a goat antiserum reared to a peptide sequence unique to cytokeratin 1 identified it on HUVEC membranes. HK bound specifically to cytokeratin only in the presence of Zn<sup>2+</sup>, as is the case of HK binding to HUVEC, and cytokeratin blocks HK binding to HUVEC (Fig. 5). Because LK binding to platelets requires Zn<sup>2+</sup>, it was postulated that the zinc requirement for kininogen binding to cells was for expression of its putative receptor (26). Unlike the interaction of HK with gC1qR (14), there is a definite zinc ion dependence in the interaction of HK with cytokeratin. Moreover, binding of HK to cytokeratin involves all the cell binding regions of HK on domains 3, 4, and 5, which have been characterized to involve binding of HK to endothelial cells (12, 13, 20, 21). This feature also distinguishes it from gC1qR, which has been characterized only to bind domain 5 of HK; LK or regions from the heavy chain of its putative receptor (26). Unlike the interaction of HK with gC1qR (14), there is a definite zinc ion dependence in the interaction of HK with cytokeratin. Moreover, binding of HK to cytokeratin involves all the cell binding regions of HK on domains 3, 4, and 5, which have been characterized to involve binding of HK to endothelial cells (12, 13, 20, 21). This feature also distinguishes it from gC1qR, which has been characterized only to bind domain 5 of HK; LK or regions from the heavy chain of its putative receptor (26).
Furthermore, there are only 2.2 × 10^5 uPAR sites on cells.

The recognition that cytokeratin 1 is a putative kininogen receptor expands our notion of the role of cytokeratins in cell biology. The fact that cytokeratin 1 can be phosphorylated suggests that this protein in modulating BK delivery and vascular biology. To date, cytokeratins are known to be part of the family of intermediate filament proteins participating in the cytoskeletal assembly of cells. No other function is known. Recognition that it is a kininogen binding protein, a putative receptor, indicates a role for this protein in modulating BK delivery and vascular biology. The fact that cytokeratin 1 can be phosphorylated suggests that kininogen binding may induce intracellular signaling (33). The discovery that cytokeratin 1 is a putative kininogen receptor in the intravascular compartment is not an isolated finding in cytokeratin biology. Recent work indicates that cytokeratin 8 is a plasminogen receptor of endothelial cells, hepatocytes, and breast cancer cells (34, 35) and that cytokeratin 18 is a binding site for thrombin–antithrombin III complexes on rabbit hepatocytes (36). These data along with our investigation suggest that some cytokeratins constitute a class of presentation receptors on cells.

We appreciate the work of Dr. Elena Ciucu on this project. This work was supported by HL55553 and H52779, and a grant-in-aid from the Michigan affiliate of the American Heart Association.