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Cell Biology

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

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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 immunoblot 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. Biotin–HK specifically bound to cytokeratin only in the presence of Zn^{2+}, 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 kininogens, 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 membranes there are multiple kininases that degrade BK once it is formed and that modulate its ability to activate its cellular receptors. BK delivery must be regulated by the binding of HK and LK to the endothelium. Identification and characterization of the kininogen receptor(s) in the intravascular compartment should contribute to our knowledge of liberation of BK and its vasoactive function.

Expression of the kininogen binding site(s) on human umbilical vein endothelial cells (HUVEC) can be modulated. First, treatment of HUVEC with metabolic inhibitors to anaerobic and aerobic metabolism and the hexose monophosphate shunt abolish the ability of HK to bind (11). Second, temperature and BK regulate the number of kininogen binding sites on HUVEC (11, 12). BK up-regulates the number of kininogen binding sites by the BK B1 receptor and a protein kinase C-mediated pathway (13). Angiotensin-converting enzyme inhibitors potentiate the effect of BK to increase expression of the HK binding site(s) on HUVEC. Third, the heavy chain of kininogens and LK have a Ca^{2+} requirement for phorbol 12-myristate 13-acetate 4-*0*-methyl ether up-regulation of their endothelial cell binding sites, whereas HK does not (13). Recently, two proteins have been identified as possible candidates for the kininogen receptor(s) on endothelial cells: gC1qR and urokinase-like plasminogen activator receptor (uPAR) (14–16). Even though gC1qR and uPAR block HK binding to HUVEC, each alone cannot fully explain the binding of kininogens 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 kininogen 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 (LDCNAEVYVVPWEKKIYPTVNCQPLGM) from domain 3 of the kininogens, MKBK (MKRPPGFSPFRSSRIG) from domain 4 of the kininogens, HKH20 (HKHGHGHGKHKNKGKKNGKH) and HVL24 (HVLDHGHKHKHGHGHGKHKNKGKK) from domain 5 of the kininogens, and FNQ15 (FNQTQPERGDNNLTR), the factor X activation peptide, were synthesized and purified in the Protein and Carbohydrate Structure Facility of the University of Michigan 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 prepared by a procedure obtained from Pierce. Peptide acetyl-RRYDQLKSDQSRLDSELC-amide (RRY16), which is a sequence unique to human cytokeratin 1 and which spans amino acid Arg_{89} to Leu_{105} (23), was synthesized and used to produce anti-peptide antisera (Anti-RRY16) in goats at Quality Control Biochemicals, Hopkinton, 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 cytokeratins 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

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Abbreviations: HK, high molecular mass kininogen; LK, low molecular mass kininogen; BK, bradykinin; HUVEC, human umbilical vein endothelial cells; uPAR, urokinase-like plasminogen activator receptor; FITC, fluorescein isothiocyanate.

*To whom reprint requests should be addressed at: University of Michigan, 5301 MSRBIII, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0640. e-mail: aschmaie@medmail.med.umich.edu.

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 (4×10^4 cells/well) were washed five times in Hepes–Tyrode's binding buffer prepared as reported (11). The cells were incubated with 20 nM biotin–HK in Hepes–Tyrode's buffer containing 50 μM Zn^{2+} at 37°C for 1 h to achieve equilibrium. Nonspecific binding was determined by measuring binding in the absence of Zn^{2+}, which is equivalent to binding seen with 50 μM Zn^{2+} 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′-tetramethylbenzidine dihydrochloride (TMB-Turbo) peroxidase substrate (Pierce) as reported (11, 12, 20, 21).

Binding of Biotin–HK to Cytokeratin. Cytokeratin (Dako) at 1 μg/well in 0.1 M Na_2CO_3 (pH 9.6) was immobilized on microtiter plates overnight at 4°C. After blocking the wells with 1% BSA for 1 h at 37°C, the wells were washed with 0.01 M sodium phosphate and 0.15 M NaCl (pH 7.4) followed by incubation of biotin–HK (20 nM) in the same buffer containing 1% BSA and 0.01% Tween 20 in the presence or absence of 50 μM Zn^{2+} with or without competitors for 1 h at 37°C. After washing, the bound biotin–HK was detected by incubation with a 1/500 dilution of ImmunoPure streptavidin horseradish peroxidase conjugate (Pierce) for 1 h followed by washing and the addition of 3,3′,5,5′-tetramethylbenzidine dihydrochloride peroxidase substrate (Pierce). Nonspecific binding was determined by the level of binding seen in the presence of a 50-fold molar excess unlabeled HK in the presence of Zn^{2+}.

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 mM 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 $12{,}000 \times g$ in a microcentrifuge to remove any particulate material. Because one endothelial cell contains 1×10^7 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 Zn^{2+} was applied to the column preequilibrated with lysate buffer containing 50 μM Zn^{2+}. 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 Zn^{2+} until the effluent OD_{280} 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

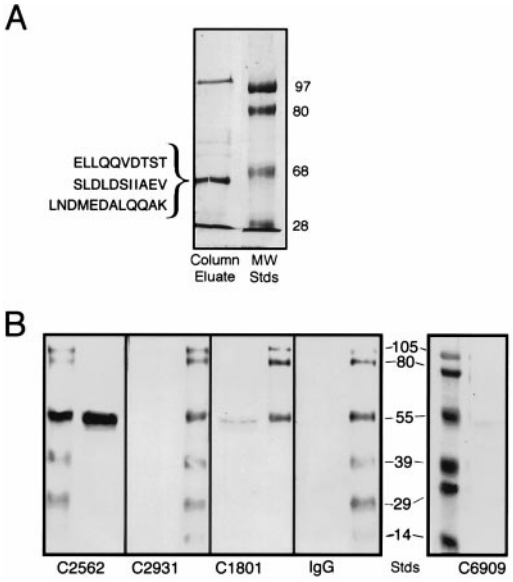

FIG. 1. (*A*) Purification of HK binding proteins. Biotin–HK was coupled to Ultralink Immobilized Streptavidin gel to prepare a HK affinity column as described in *Experimental Procedures*. HUVEC lysates were applied to the column and bound protein was eluted with 0.2 M glycine (pH 2.8) (Column Eluate). The letters to the left of the left lane represent three amino acid sequences from two separate occasions obtained from tryptic digests of the shown 54-kDa band. The lane and numbers on the right represent molecular mass standards (MW Stds) in kilodaltons. The figure is a photograph of a Coomassie blue R-250-stained 8% SDS/PAGE. (*B*) Immunoblot of HUVEC lysates with anti-cytokeratin antibodies. HUVEC lysates were prepared as indicated in *Experimental Procedures*. After electrophoresis of the lysate on 11% SDS/PAGE, the samples were transferred to nitrocellulose. Strips containing lysate were cut out and placed individually in containers containing mAbs C2562, C2931, C1801, mouse IgG, or C6909 in 0.01 M sodium phosphate and 0.15 M NaCl, (pH 7.4) containing 1% BSA and 0.01% Tween 20. After each strip was incubated and washed, the nitrocellulose was incubated with a rabbit anti-mouse antibody conjugated with horseradish peroxidase and then developed with 4-chloro-1-naphthol substrate. The antibody name is placed under the lane that characterizes the presence or absence of a band specific for cytokeratin 1. The numbers between the two photographs of the nitrocellulose membrane represent molecular mass standards in kilodaltons. The other stained lanes in each panel represent molecular mass standards.

visualized with Coomassie blue R-250. Amino acid sequencing of the isolated material was performed by Joseph Leykam at the Macromolecular Structure Facility of Michigan State University, East Lansing, MI. Because the isolated bands were blocked at the N terminus, tryptic digestion of the bands was performed. Trypsin was added to the protein at ≈4% wt/wt by estimating the amount of protein from the gel (usually 150–250 ng of trypsin). The reaction proceeded for 18–20 h at 37°C. After stopping the reaction by adding an equal volume of 0.25% trifluoroacetic acid, the tryptic digests of each band were separated on a 0.8×250-mm C-18 HPLC column by using a trifluoroacetic acid-acetonitrile gradient. Amino acid N-terminal sequencing was performed on an Applied Biosystems model 494 protein/peptide sequencer. Identified sequences were analyzed by comparison to known protein sequences in the GenBank database.

Immunoblot Studies. Immunoblotting of HUVEC lysates was performed on samples electrophoresed by 11% SDS/PAGE. After transfer to nitrocellulose, the membrane was blocked with Blotto, which consisted of 5% wt/vol nonfat dry milk in 0.01 M sodium phosphate and 0.15 M NaCl (pH 7.4) (24). After blocking, the membranes were incubated with a primary anti-cytokeratin mAb in 0.01 M sodium phosphate and 0.15 M NaCl (pH 7.4) containing 1% BSA and 0.01% Tween 20. After washing, a secondary rabbit anti-mouse antibody conjugated with horseradish peroxidase was added.

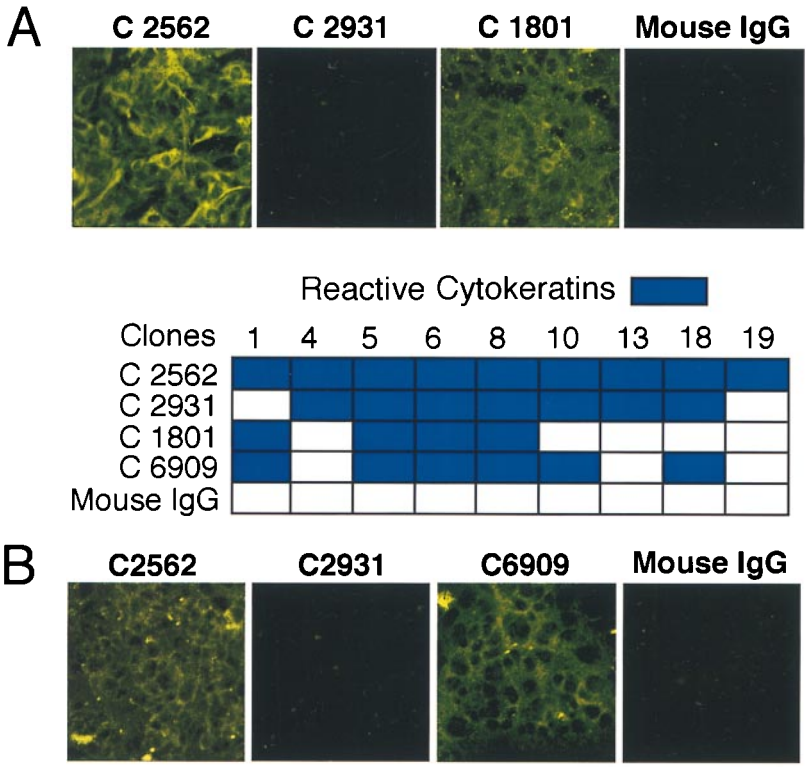

FIG. 2. Laser scanning confocal microscopy. (*A*) Paraformaldehyde (2%) fixed but nonpermeabilized HUVEC grown on microscope slides were incubated with mAbs C2562, C2931, C1801, and mouse IgG. (*B*) Unfixed and nonpermeabilized HUVEC grown on microscope slides were incubated with mAbs C2562, C2931, C6909, 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.

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 × *g* for 5 min and washed with Hepes–Tyrode's binding buffer. Cells (2×10^5) 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 × *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 ≥15,000 HUVEC was analyzed in each sample.

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 Hepes–Tyrode's buffer, the cells were incubated for 1 h at room temperature with 1 μg/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).

RESULTS

Purification of HK Binding Proteins. The biotin–HK immobilized streptavidin affinity column repeatedly isolated 54- and 102-kDa proteins from HUVEC lysates (Fig. 1*A*). On occasion,

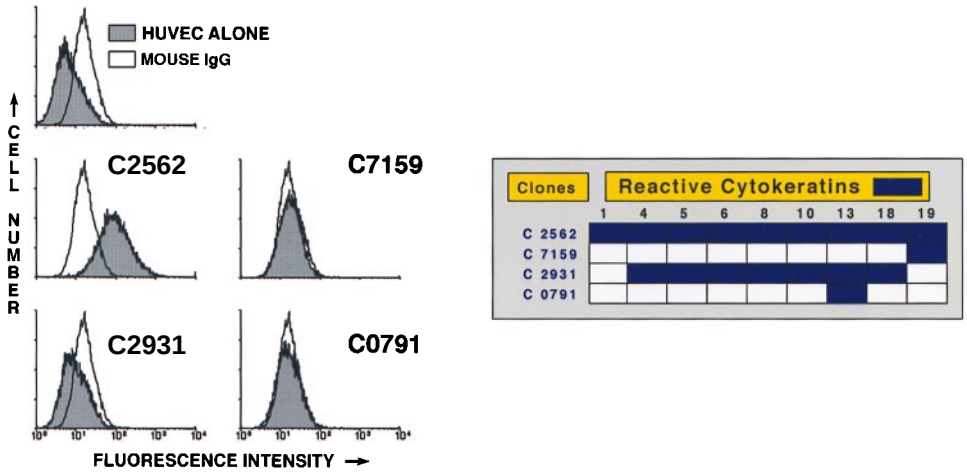

FIG. 3. Flow cytometry of HUVEC. Suspensions of washed, unfixed, and nonpermeabilized HUVEC were incubated with mouse IgG (unshaded curves) or mAbs C2562, C7159, C2931, or C0791, each added at 1/100 dilution. The binding of these antibodies on the HUVEC membrane was detected with a secondary antibody 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.

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, SLDLDSIIAEV and LNDMEDALQQAK, were obtained and were identified as cytokeratins including cytokeratin 1. The sequence SLDLDSIIAEV is found in cytokeratins 1, 6, 5, 8, and 4 and is coded by exon 5 of the gene for cytokeratin 1 (23) (Fig. 1*A*). The sequence LNDMEDALQQAK 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 ELLQQVDTST 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. 1*B*). 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 pancytokeratins 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. 2*A*). 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. 2*A*). Further investigations were performed to determine whether cytokeratin antigen could be detected on the membranes of unfixed and nonpermeabilized HUVEC (Fig. 2*B*). 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 (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 (R_{89}RYDQLKSDQSRLDSEL_{105}C) 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 antiserum recognized cytokeratin 1 on the membrane of unfixed and nonpermeabilized HUVEC in suspension (Fig. 4*A*). Cytokeratin 1 antigen also was present in a purified cytokeratin preparation purchased from Dako (Fig. 4*B*). When anti-RRY16 antiserum 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. 4*B*).

Interactions Between HK and Cytokeratin 1. Investigations next were performed to determine whether biotin–HK bound to purified cytokeratin (Fig. 5*A*). 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. 5*B*). Increasing concentrations of purified cytokeratin (2 pM to 3 μM) blocked biotin–HK binding with an apparent IC_{50} 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. 6*A*). Increasing concentrations of HK and LK but not factor XII blocked biotin–HK binding to cytokeratin (Fig. 6*A*). HK and LK blocked biotin–HK binding to

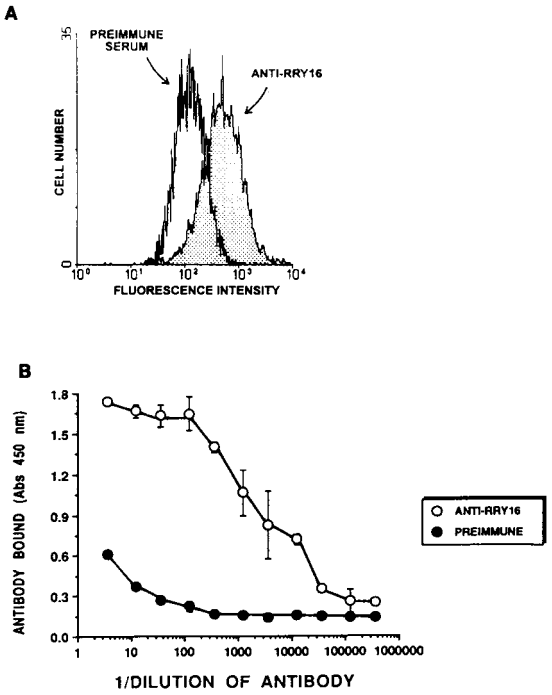

FIG. 4. (*A*) Flow cytometry with anti-RRY16 antisera and its preimmune serum. Washed HUVEC in suspension in Hepes–Tyrode's buffer were incubated with 1/100 dilution of anti-RRY16 antiserum or preimmune serum for 1 h at 4°C. After centrifugation and resuspension in Hepes–Tyrode's buffer, they were incubated with a mouse anti-goat antibody conjugated with FITC. The flow cytogram shown is a representative study of two cytograms. (*B*) Binding of anti-RRY16 antisera or its preimmune serum to cytokeratin. Purified cytokeratin (1 μg/well) was coupled to microtiter plate wells in 0.1 M Na_2CO_3 (pH 9.6) overnight at 4°C. After washing the wells with 0.01 M sodium phosphate and 0.15 M NaCl (pH 7.4), the indicated dilution of anti-RRY16 antiserum or its preimmune serum was added to the microtiter plate wells. The presence of antibody bound to the cytokeratin-coated wells was detected by using a mouse anti-goat antibody conjugated with peroxidase and peroxidase substrate. The data presented are the mean ± SEM of three determinations at each dilution.

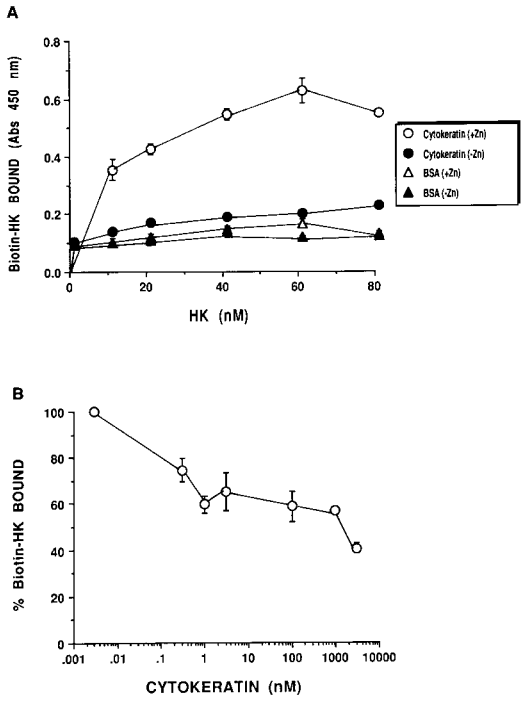

FIG. 5. HK and cytokeratin interactions. (*A*) Microtiter plates were coated in 0.1 M Na_2CO_3 buffer (pH 9.6) with a purified cytokeratin mixture or BSA both at 1 μg/well. The ability of HK to bind to cytokeratin was determined by adding increasing concentrations of biotin–HK (2–80 nM) to wells coated with cytokeratin or BSA in the absence (−Zn) or presence (+Zn) of 50 μM Zn^{2+}. The data presented are the mean ± SEM of three individual experiments at each point. (*B*) Investigation to determine whether cytokeratin blocks HK binding to HUVEC. Increasing concentrations of purified cytokeratin (0.002–3,000 nM) were incubated with biotin–HK (20 nM) in Hepes–Tyrode's buffer containing 50 μM Zn^{2+} over confluent HUVEC in microtiter plates. After incubation for 1 h, the cells were washed and the degree of biotin–HK bound to the HUVEC was determined by procedures reported in *Experimental Procedures.* Each point is the mean ± SEM of three determinations.

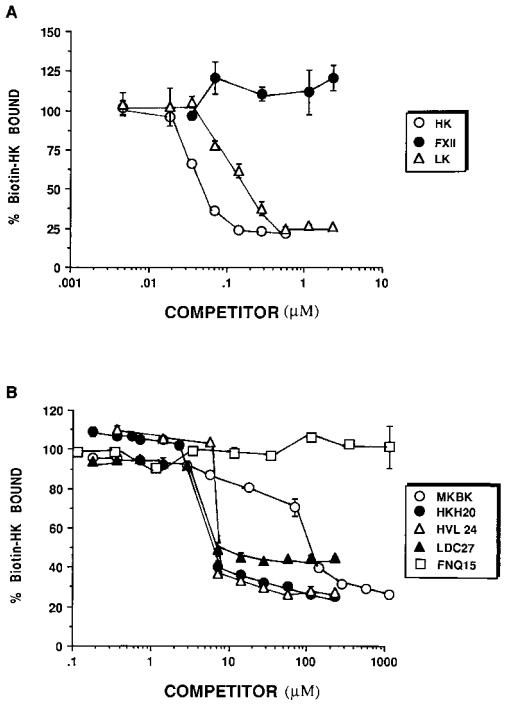

FIG. 6. (*A*) Inhibition of biotin–HK binding to cytokeratin. Biotin–HK (20 nM) in the absence or presence of increasing concentrations of purified HK, LK, or factor XII was incubated in 0.01 M sodium phosphate and 0.15 M NaCl (pH 7.4) containing 1% BSA, 0.01% Tween 20, and 50 μM Zn^{2+} in microtiter plate wells that were coated with 1 μg/well cytokeratin. The amount of biotin–HK bound to the microtiter plate wells was determined as indicated in *Experimental Procedures.* The data presented are the mean ± SEM of three experiments. (*B*) Biotin–HK (20 nM) in the absence or presence of increasing concentrations of peptides MKBK from domain 4 of kininogens, HKH20 and HVL24 from domain 5 of HK, LDC27 from domain 3 of kininogens, or a control peptide, FNQ15, was incubated in 0.01 M sodium phosphate and 0.15 M NaCl (pH 7.4) containing 1% BSA, 0.01% Tween 20, and 50 μM Zn^{2+} in microtiter plate wells that were coated with 1 μg/well cytokeratin. The amount of biotin–HK bound to the microtiter plate wells was determined as indicated in *Experimental Procedures.* The data presented are the mean ± SEM of three experiments.

cytokeratins with an IC_{50} of 40 nM and with an IC_{50} 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. 6*B*). 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_{50} of 100 μM for MKBK and with an IC_{50} 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 from 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, immunofluoresence, 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^{2+}, 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^{2+}, 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 the kininogens do not bind to gC1qR (14).

The recognition that cytokeratin 1 is a kininogen binding protein enlarges the list of possible kininogen receptors on endothelial cells and, perhaps, other cells in the intravascular compartment. The protein, gC1qR, which is the known receptor for C1 (27), binds HK, and its binding is competed by factor XII (14, 15). This interaction is consistent with the finding that factor

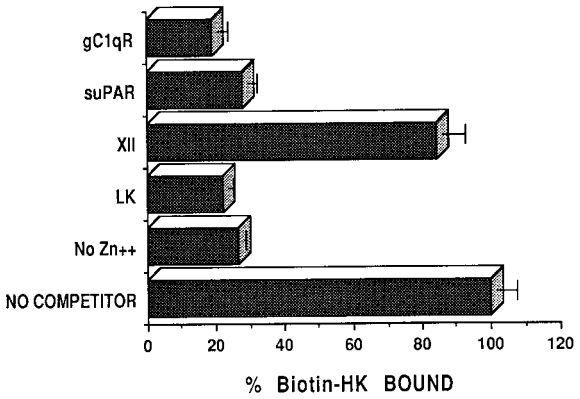

FIG. 7. Influence of other proteins on biotin–HK binding to cytokeratin. Biotin–HK (20 nM) in the absence (NO COMPETITOR) or presence of 1 μM of LK, factor XII (XII), soluble uPAR, or gC1qR fusion protein was incubated in 0.01 M sodium phosphate and 0.15 M NaCl (pH 7.4) containing 1% BSA, 0.01% Tween 20, and 50 μM Zn^{2+} in microtiter plate wells that were coated with 1 μg/well cytokeratin. Nonspecific binding (No Zn^{2+}) was determined by biotin–HK binding in the absence of added Zn^{2+}. The amount of biotin–HK bound to the microtiter plate wells was determined as indicated in *Experimental Procedures.* The data presented are the mean ± SEM of three experiments.

XII blocks HK binding to HUVEC (28). However, gC1qR cannot be the only kininogen receptor because a limited amount of it is present on HUVEC, it only binds HK and not LK, and its binding to HK is not Zn^{2+}-dependent (14, 17, 18). The urokinase plasminogen activator receptor (uPAR) also is another candidate for kininogen receptor on HUVEC, because it has been reported to block HK binding to HUVEC (16). However, its absence from the membrane of platelets, which also binds kininogens (19, 29, 30), removes it as the single candidate for the kininogen receptor. Furthermore, there are only 2.2×10^5 uPAR sites/endothelial cell vs. 1–10 $\times$ 10^6 binding sites for kininogens (11, 31). The finding that the fusion protein of gC1qR with maltose binding protein and soluble uPAR blocked binding of HK to cytokeratin indicates that these proteins interact with the same region(s) on HK that allow HK to bind to cytokeratin. On endothelial cells, kininogens could be associating with a multiprotein receptor complex that includes cytokeratin 1, uPAR, and gC1qR. The finding that factor XII did not block HK's binding to cytokeratin indicates that the interaction of factor XII with HK must be mediated through the membrane expression of gC1qR and not cytokeratin 1. Because factor XIIa activates the classic complement pathway, this interaction with gC1qR is not surprising (14, 15, 32). It suggests that the role of gC1qR in the multiprotein kininogen receptor complex is to modulate factor XII binding to endothelial cells to regulate the activation of the classic complement pathway.

The recognition that cytokeratin 1 is a putative kininogen receptor expands our notion of the role of cytokeratins in cell 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.

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