Human plasminogen contains structural domains that are termed kringlees. Proteolytic cleavage of plasminogen yields kringlees 1–3 or 4 and kringle 5 (K5), which regulate endothelial cell proliferation. The receptor for kringlees 1–3 or 4 has been identified as cell surface- associated ATP synthase; however, the receptor for K5 is not known. Sequence homology exists between the plasminogen activator streptokinase and the human voltage- dependent anion channel (VDAC); however, a functional relationship between these proteins has not been reported. A streptokinase binding site for K5 is located between residues Tyr252–Lys283, which is homologous to the primary sequence of VDAC residues Tyr252–Lys283. Antibodies against these sequences react with VDAC and detect this protein on the plasma membrane of human endothelial cells. K5 binds with high affinity (Kd of 28 nM) to endothelial cells, and binding is inhibited by K5 also interferes with mechanisms controlling the regulation of intracellular Ca2+ via its interaction with VDAC. K5 binding to endothelial cells also induces a decrease in intracellular pH and hyperpolarization of the mitochondrial membrane. These studies suggest that VDAC is a receptor for K5.

Angiogenesis is essential for tumor growth (1–4). Vascular endothelial growth factor (VEGF)1 is a potent mitogen promoting endothelial cell proliferation (5, 6), whereas angiostatin (San Diego, CA). Endothelial cell growth supplement was from Collaborative Research Inc. (Waltham, MA).125I-Labeled Bolton-Hunter reagent was obtained from PerkinElmer Life Sciences. The 21-amino acid sequence EINNTDLISLEYKYVLKKGEK (Glu263–Lys283) of SK and KVNNSSLIGLYTQLPKGIK (Lys265–Lys285) of VDAC1 were from Research Genetics (Huntsville, AL). Fura-2/AM and bis(carboxyethyl)- carbonyl fluorescein and the 2-(dimethylamino)styryl-1-methyl- pyridinium ion (DSPM+) were purchased from Molecular Probes, Inc. (Eugene, OR).

**Proteins—** Human Pg was resolved into its isoforms, Pg 1 and 2 (17, 18). Pg 2 was digested with elastase and fractionated by gel and affinity chromatography to obtain mini-Pg; followed by digestion of mini-Pg with pepsin to obtain K5 (10, 19, 20). Gel electrophoresis (10–20% gradient gel, nonreducing conditions) identified a doublet of ~12 kDa, which was identified by mass spectrometry as K5. Amino-terminal sequence analysis yielded the sequence LPTVETPSEE, corresponding to Pg residues 450–459, confirming the identification of K5 (21). Reduction/alkylation of K5 was performed by incubating 20 μg of K5 with 1 m mol dithiothreitol for 30 min followed by incubation with 5 m mol iodoacetamide for 30 min, both at room temperature, and removal of these reagents by dialysis versus 10 m mol Hepes, pH 7.5. Ionization of K5 was performed with 125I-labeled Bolton-Hunter reagent (specific activity, 500–700 cpm/pg).

**Antibodies—** Antibodies to SK were raised in rabbits, and the IgG fraction specific for the SK sequence Glu273–Lys279 was purified by immunoaffinity on a resin containing this peptide conjugated to activated carboxyhexyl-Sepharose (Amersham Biosciences). The antibodies against the 21-amino acid sequence Lys265–Lys285 of VDAC1 conjugated to keyhole limpet hemocyanin (22) were prepared in rabbits by COVANCE (Denver, PA). The IgG fraction specific for VDAC1 was purified by immunoaffinity on a resin containing the VDAC1 peptide conjugated to carboxyhexyl-Sepharose. The monoclonal antibody 2B12 against human mitochondrial VDAC1 was from Molecular Probes, Inc. **Endothelial Cell Proliferation Assay—** HUVEC from Clonetics (San Diego, CA) were grown in Dulbecco’s modified Eagle’s medium containing 20% bovine serum, 100 units/ml penicillin/streptomycin, 2 μg/ml amphotericin B, 2 m mol glutamine, 5 units/ml sodium heparin, and 200 μg/ml endothelial cell growth supplement (23). The cells were washed with phosphate-buffered saline and dispersed in a 0.05% trypsin solu-
tion. The cells were resuspended in medium (25 × 10^6 cells/ml) and plated in 96-well culture plates (0.2 ml/well). After 24 h at 37 °C, the medium was replaced with 0.2 ml of Dulbecco’s modified Eagle’s medium, 5% bovine serum, 1% antibiotics, and the test samples were applied. Cell proliferation was determined at 24 h using bromodeoxyuridine labeling and a colorimetric immunoassay (Roche Applied Science). The results were expressed as percentages of control proliferation determined in the presence of VEGF (10 ng/ml) and the absence of K5.

**Flow Cytometry**—HUVEC were detached from the culture flasks (75 cm²) by incubation for 5 min at 37 °C with Ca²⁺ and Mg²⁺-free phosphate-buffered saline containing 4 mM EDTA and pelleted. The cells (1 × 10⁶/ml) were washed with phosphate-buffered saline before resuspension in ice-cold Phenol Red-free Hanks balanced salt solution (HBSS), 1% BSA, 0.3 mg/ml goat IgG, and 0.01% NaN₃ (staining buffer). The cell suspensions (100 μl) were incubated 30 min with dilutions of rabbit polyclonal anti-human SK peptide IgG, anti-human VDAC1 peptide IgG, or the murine anti-human mitochondrial VDAC1 monoclonal antibody. The cells were washed with ice-cold staining buffer, pelleted, and resuspended in 100 μl of ice-cold staining buffer. The cell suspensions were incubated in the dark with an AF488-conjugated for 30 min to goat anti-rabbit or mouse IgG from Molecular Probes, Inc. The cells were rinsed with RPMI 1640, and the wells stripped after obtaining a stable baseline (25).

**Gel Electrophoresis**—Electrophoresis was performed in 0.1% SDS modified Eagle’s medium on glass coverslips and then washed with HBSS with 0.1 mM sodium bicarbonate, pH 7.1. The cells were incubated for 20 min with 2 μM 2,7-bis-(2-carboxethyl)-5-(and -6)-carboxyfluorescein (BCECF) in HBSS, rinsed with buffer thrice, and placed on the fluorescent microscope stage. Intracellular pH (pHi) was measured by a digital video imaging technique in cells stimulated by the ligands, which were added after obtaining a stable base line (25).

**Antibody Binding Studies**—The binding assays were performed in HUVEC grown in 96-well strip plates. The cells were washed in HBSS and incubated with increasing concentrations of 125I-labeled anti-human VDAC1 peptide IgG for 90 min at 25 °C in RPMI 1640 containing 2% BSA. The cells were rinsed with RPMI 1640, and the wells stripped from the plates were inserted in plastic tubes to determine radioactivity. IgG bound was calculated after subtraction of nonspecific binding measured in the presence of 50 μM unlabeled IgG. The Bmax of the anti-VDAC1 IgG was then calculated.

**Measurements of Intracellular Free Ca²⁺ Concentration and Cytosolic pH**—HUVEC [Ca²⁺]i, was measured by digital imaging microscopy using the fluorescent indicator Fura-2/AM (24). For measurements of pHi, HUVEC were incubated overnight in Dulbecco’s modified Eagle’s medium on glass coverslips and then washed with HBSS with 0.1 mM sodium bicarbonate, pH 7.1. The cells were incubated for 20 min with 2 μM 2,7-bis-(2-carboxylethyl)-5-(and -6)-carboxyfluorescein (BCECF) in HBSS, rinsed with buffer thrice, and placed on the fluorescent microscope stage. Intracellular pH (pHi) was measured by a digital video imaging technique in cells stimulated by the ligands, which were added after obtaining a stable base line (25).

**Gel Electrophoresis**—Electrophoresis was performed in 0.1% SDS employing a discontinuous Laemmli buffer system (26). The gels were stained with 0.25% Coomassie Brilliant Blue R-250. Transfer to nitrocellulose membranes was carried out by the Western blot method (27). The dye-conjugated markers (Bio-Rad) used were of Mr, 38,100, 28,400, 18,200, 9,200, and 4,300.

**Purification of VDAC1 From 1-LN Cells**—It is difficult to obtain large numbers of cultured HUVEC; however, we found that 1-LN cells are a good source of this protein. 1-LN cells were grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin G, and 100 ng/ml streptomycin in 20 culture flasks (150 cm²). After detaching with 10 mM EDTA in HBSS and pelleting, the cells were suspended in 10 ml of 20 mM Hepes, pH 7.2, 0.25% sucrose containing the proteinase inhibitors (each at 0.5 mg/ml) antipapain, bestatin, chymostatin, trans-epoxysuccinyl-1-leucylamido- (4-guanidino)butane (E-64), leupeptin, pepstatin, o-phenanthroline, and aprotinin. The cells were lysed by sonication on ice (five 10-s bursts with 30-s intervals). The homogenate was centrifuged at 800 × g for 15 min, followed by centrifugation at 50,000 × g for 1 h. The pellet containing cell membranes was resuspended in 20 mM Tris-HCl, pH 8.0, containing 1% (v/v) Triton X-100.
Fig. 2. Binding of K5 to VDAC1 incorporated into proteoliposomes. All of the experiments were performed by gel filtration on a Sephadex G-75 column (55 × 2 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, at room temperature. A, 125I-K5 (30 ng) filtered through the column. B, 125I-K5 (30 ng) reacted with VDAC1 (2 μg) and then filtered through the column. C, VDAC1 (2 μg) incubated with preassembled proteoliposomes in 5% MeSO with agitation for 30 min. At this time, the concentration of MeSO was reduced to 0.5% by addition of 125I-K5 (30 ng) in 50 mM Tris-HCl, pH 7.5, incubated with agitation for another 30 min, followed by gel filtration. D, conditions were identical to those in C except that the mixture of VDAC1 and proteoliposomes was incubated for 30 min with a rabbit anti-VDAC1 (peptide Lys235–Lys255) IgG before the addition of 125I-K5 and gel filtration.

X-100 to solubilize membranes and centrifuged at 50,000 × g for 30 min to remove insoluble materials. VDAC was sequentially purified to homogeneity using gel filtration on Sephadex G-150 and immunoaffinity chromatography with an anti-VDAC peptide IgG conjugated to Sepharose 4-B (see Fig. 1C).

Fig. 3. Specific binding of K5 to VDAC1 proteoliposomes. A, increasing concentrations of 125I-K5 were incubated with VDAC1 proteoliposomes (●), BSA proteoliposomes (○), or empty liposomes (△) (10 μM phospholipid each) in 2.5 mM Hepes, pH 7.4, 145 mM NaCl. B, inhibition of 125I-K5 (100 nM) binding to VDAC1 proteoliposomes (10 μM phospholipid) by increasing concentrations of nonlabeled K5 (●) or anti-VDAC1 IgG (△). The data are the means ± S.D. from experiments performed in triplicate.

X-100 to solubilize membranes and centrifuged at 50,000 × g for 30 min to remove insoluble materials. VDAC was sequentially purified to homogeneity using gel filtration on Sephadex G-150 and immunoaffinity chromatography with an anti-VDAC peptide IgG conjugated to Sepharose 4-B (see Fig. 1C).

Incorporation of VDAC1 into Liposomes and Binding of K5 to the Reconstituted Receptor—Purified VDAC1 was reconstituted into liposomes (28, 29) as follows. 50 μl of a suspension of liposomes (8 μM 1-O-phosphatidylcholine, 8 μM phosphatidylethanolamine, 8 μM β-oleoyl-γ-palmitate and 6.9 μM cholesterol) in 5% MeSO were mixed with VDAC1 (5 μg) and incubated with agitation for 30 min at room temperature. The concentration of MeSO was reduced to 0.5% with 50 mM Tris-HCl, pH 7.4. After the addition of 125I-K5 (10 nM) and incubation for another 30 min at room temperature, the mixture was filtered through a Sephadex G-75 column (55 × 2 cm). To study inhibition of K5 binding to VDAC1 reconstituted into liposomes, the mixture was incubated with the specific anti-VDAC1 IgG for 30 min at room temperature before the addition of 125I-K5.

The kinetic parameters of K5 binding to VDAC1 on reconstituted liposomes were performed on large unilamellar liposomes (0.4 μm in diameter) prepared by extrusion of multimamellar vesicles through 0.4-μm defined polycarbonate filters (Nucleopore, Pleasanton, CA) (30). For these experiments, proteoliposomes containing VDAC1 or BSA were prepared by mixing the proteins (50 μg) in 2.5 mM Hepes, pH 7.4, 145 mM NaCl, and 0.3 mM N-dodecyl-β-D-maltopyranoside with N-dodecyl-β-D-maltopyranoside saturated (0.6 mM) liposomes at a 1.3 volume ratio of protein preparations to liposomes (31). The detergent was removed after three 2-h incubations at 4 °C of the proteoliposomes with 10 mg of Biobeads SM2 (Bio-Rad) followed by three 30-min centrifugations at 100,000 × g. Phospholipid phosphate was then determined using a phospholipid phosphate assay kit (NEN). Phospholipid phosphate was then determined using a phospholipid phosphate assay kit (NEN).
phospholipid) in 2.5 mM Hapes, pH 7.4, containing 145 mM NaCl. Filtration and determination of kinetic parameters were carried out as described above.

Preparation of Mitochondria—Mitochondria from 1-LN cells were isolated (33), and the protein levels were estimated using the bicinchoninic acid method (34).

Mitochondrial Membrane Potential—Membrane potential (Δψ) was determined at room temperature using DSMP+, a fluorescent indicator of membrane potential (35). The assay consisted of a final volume of 2 ml containing sucrose (250 mM), Hapes (10 mM), EGTA (2.5 mM) pH 7.4, mitochondria (0.2 mg) cellular protein, DSMP + 2 (nmol), rotenone (1 μg), sodium succinate (10 mM), pH 7.4, and increasing concentrations of K5. The mixture was incubated for 20 min with K5 prior to addition of DSMP+. The fluorescence intensity was measured (excitation λ = 489 nm, emission λ = 566 nm) in a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Corporation, Kyoto, Japan). Maximal fluorescence in the absence of K5 was obtained with mitochondria incubated with DSMP+ alone. The results are the means of fluorescence determinations from three experiments.

RESULTS

Analyses of Sequence Similarities between SK and Human VDAC1—The regions of sequence similarity between streptokinase and human VDAC1 were identified by the BLAST program provided by the Swiss Institute of Bioinformatics (Fig. 1A). The topology prediction for helical transmembrane proteins was solved with use of the hidden Markov model, also provided by the Swiss Institute of Bioinformatics, and shows a loop through the outer mitochondrial membrane spanning VDAC1 residues 247–255 (Fig. 1B). We raised rabbit antibodies to the SK peptide EINNTDLISLEYKV-LKKGEK283 and the VDAC1 peptide KVNVSLSLGY-TQTLKPGIK255. A Coomassie Brilliant Blue stain of the purified VDAC1 showed reactivity with p-VDAC1 (peptide Glu263–Lys283) IgG (Fig. 1C, lane 2), confirming the structural relatedness between VDAC1 and SK. However, the purified VDAC1 did not show any reactivity with 125I-K5 when electroblotted to a nitrocellulose membrane (Fig. 1C, lane 4).

Binding of K5 to VDAC1 Incorporated into Liposomes—The experiments described above demonstrate a significant impact on the ability of purified receptor to bind to K5; therefore, the purified VDAC1 was incorporated into liposomes and gel filtration on Sephadex G-75 employed to identify and separate the reactants (Fig. 2). 125I-K5 eluted at a column volume of 100–120 ml (Fig. 2A). When 125I-K5 was incubated with solubilized VDAC1 (2 μg), the radiolabeled material eluted in the same fractions as above, suggesting no reactivity between K5 and solubilized receptor (Fig. 2B). When VDAC1 was incorporated into liposomes and then reacted with K5, the radiolabeled material eluted from the column as two peaks, one of them corresponding to the void volume where VDAC1 elutes and the other corresponding to the elution volume of unreacted K5 (Fig. 2C). These data indicate that K5 binds to VDAC1 when this

FIG. 5. A, anti-endothelial cell proliferative activity. Increasing concentrations of native K5 (●) and reduced/alkylated K5 (▲) were assayed in the presence of VEGF (10 ng/ml) in a 24-h proliferation assay. The inhibitory effect of K5 is expressed as a percentage of control cells grown only in the presence of VEGF. B, binding of K5 to HUVEC. Increasing concentrations of 125I-K5 were added to HUVEC monolayers (2 × 10⁴ cells/well). Bound ligand was calculated after subtraction of nonspecific binding measured in the presence of 50 μM p-amino-benzamidine. Inset, blot binding assay of a SDS/10% PAGE (nonreducing conditions) of HUVEC lysate incubated with anti-VDAC1 IgG. The data are the means ± S.D. from experiments performed in triplicate. C, inhibition of binding of K5 to HUVEC. 125I-K5 (100 nM) was incubated with increasing concentrations of nonlabeled Fg (■), kringles 1–3 angiostatin (○), mini-Pg (△), K5 (●), or anti-VDAC1 (peptide Lys235–Lys255) IgG (▲). D, binding of 125I-labeled anti-VDAC1 (peptide Lys235–Lys255) IgG to HUVEC. Increasing concentrations of IgG were added to HUVEC cell monolayers (2 × 10⁴ cells/well). IgG bound were calculated after subtraction of nonspecific binding measured in the presence of nonlabeled anti-VDAC1 IgG (50 μM). The data are the means ± S.D. from experiments performed in triplicate.
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Effect of K5 Binding on HUVEC—K5 binds to these cells in a dose-dependent manner with high affinity (K<sub>i</sub> of 22 ± 3.1 nm). The binding is specific for VDAC1 because control proteoliposomes prepared with BSA or empty liposomes show little specific binding (Fig. 3A). Binding of 125I-K5 to VDAC1 proteoliposomes is inhibited by unlabeled K5 or anti-VDAC1 IgG (Fig. 3B), suggesting that VDAC1 is a receptor for K5.

Analyses of VDAC1 on the Cell Surface of HUVEC by Flow Cytometry—As determined by FACS, HUVEC reacted with an antibody against the SK peptide (Fig. 4A) as well as an antibody against the VDAC1 peptide (Fig. 4B) or a murine antibody against human mitochondrial VDAC1 (Fig. 4C), as expected because mitochondrial and plasma membrane VDAC1 share the same primary structure (35). FACS analysis of HUVEC reacted with K5 (0.1 μM) prior to reaction with antibody against the VDAC1 peptide (Fig. 4D) shows inhibition of binding of this antibody, suggesting that both K5 or the IgG compete for the same binding site. Taken together, these experiments show that VDAC1 is not only expressed on the surface of HUVEC but also establishes the structural relationship between SK and VDAC1 hypothesized by McCabe et al. (16).

Inhibition of Endothelial Cell Proliferation by K5—K5 inhibited VEGF-dependent HUVEC proliferation in a dose-depend-ent manner (Fig. 5A). As observed previously (10), the anti-endothelial cell proliferation of K5 was abolished after reduction/alkylation of the protein, suggesting that the formation of appropriate disulfide bridges is essential to maintain its activity.

Binding of K5 to HUVEC—K5 binds to these cells in a dose-dependent manner with high affinity (K<sub>i</sub> of 28 ± 1.37 nm) and to a large number of sites (12.6 ± 0.56 × 10<sup>5</sup> binding sites/cell) (Fig. 5B). The value of the K<sub>i</sub> is comparable with that determined for binding of K5 to VDAC1 reconstituted in proteoliposomes. Electrophoretic separation of proteins in a HUVEC lysate followed by a blot binding assay with a rabbit anti-VDAC1 IgG (Fig. 5B, inset) shows only one band of M<sub>r</sub> ∼32,000. Binding of K5 to HUVEC is inhibited by Pg, Pg peptides containing K5, or by an IgG fraction against VDAC1 peptide showing structural relatedness to SK (Fig. 5C).

Binding of Anti-VDAC1 Peptide IgG to HUVEC—125I-La-
ed anti-VDAC1 IgG bind to HUVEC in a dose-dependent manner to a large number of sites (B<sub>max</sub> of 11.6 × 10<sup>5</sup> binding sites/cells) (Fig. 5D). This value is comparable with that determined for the binding of K5 to HUVEC, suggesting VDAC1 as a unique receptor for K5 on the cell surface.

Effect of K5 Binding on HUVEC [Ca<sup>2+</sup>] i and pH<sub>i</sub>—We also investigated whether K5 binding to HUVEC produced changes in [Ca<sup>2+</sup>] i or pH<sub>i</sub> and compared these changes with those produced by Pg 2. Pg 2 (100 nM) added to HUVEC induces a transient rise in [Ca<sup>2+</sup>] i lasting about for 90 s before returning to base line (Fig. 6A). Pg 2 also induced a rise in pH<sub>i</sub>, which was continuous for 400 s (Fig. 6B). A similar concentration (100 nm) of K5 induced a small a rise in [Ca<sup>2+</sup>] i (Fig. 6C) and produced a continuous decrease in pH<sub>i</sub> during the same time period (Fig. 6D). Incubation of HUVEC with K5 followed by Pg 2 shows a decreased stimulation in [Ca<sup>2+</sup>] i (Fig. 6E); however, the decrease in pH<sub>i</sub> induced by K5 is abolished after the addition of Pg (Fig. 6F). Incubation of HUVEC with anti-VDAC1 peptide IgG prior to the addition of K5 causes no change in [Ca<sup>2+</sup>] i (Fig. 6G) or pH<sub>i</sub> (Fig. 6H and Table 1).

Effect of K5 on Mitochondrial Membrane Potential—The flu-

![Fig. 7. Effect of K5 on 1-LN cell mitochondrial potential. Δψ was determined using DSPM+, a fluorescent indicator of membrane potential. Mitochondria were incubated with increasing concentrations of K5 for 20 min prior to addition of DSPM+. The fluorescence intensity was monitored at an excitation λ = 485 nm and an emission λ = 566 nm. The data are the means ± S.D. from experiments performed in triplicate.](http://www.jbc.org/)

### Table 1

| Ligand       | Changes in [Ca<sup>2+</sup>]<sub>i</sub> | Changes in pH<sub>i</sub> |
|--------------|----------------------------------------|--------------------------|
| Pg 2         | +450                                   | +0.17                    |
| K5           | +100                                   | –0.20                    |
| K5 + Pg2     | +10                                    | +0.07                    |
| K5 + anti-VDAC1 | +10                               | +0.07                    |

These data were obtained from Fig. 6.
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tient of Tyr512 is required for reactivity of K5 with its nated techniques producing tyrosine modification of K5. The ever, no specific binding of K5 to endothelial cells was detected endo endothelial cells (43). Cell proliferation is also dependent on cytosolic pH

3) does not affect endothelial cell proliferation or –

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