Tuftsin Binds Neuropilin-1 through a Sequence Similar to That Encoded by Exon 8 of Vascular Endothelial Growth Factor*

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Tuftsin, Thr-Lys-Pro-Arg (TKPR), is an immunostimulatory peptide with reported nervous system effects as well. We unexpectedly found that tuftsin and a higher affinity antagonist, TKPPR, bind selectively to neuropilin-1 and block vascular endothelial growth factor (VEGF) binding to that receptor. Dimeric and tetrameric forms of TKPPR had greatly increased affinity for neuropilin-1 based on competition binding experiments. On endothelial cells tetrameric TKPPR inhibited the VEGF165-induced autophosphorylation of vascular endothelial growth factor receptor-2 (VEGFR-2) even though it did not directly inhibit VEGF binding to VEGFR-2. Homology between exon 8 of VEGF and TKPPR suggests that the sequence coded for by exon 8 may stabilize VEGF binding to neuropilin-1 to facilitate signaling through VEGFR-2. Given the overlap between processes involving neuropilin-1 and tuftsin, we propose that at least some of the previously reported effects of tuftsin are mediated through neuropilin-1.

Tuftsin, a naturally occurring peptide with the sequence TKPR, was originally described in 1970 by Najjar and Nishioka (1) as a phagocytosis-stimulating peptide derived from the proteolytic degradation of IgG. Subsequent reports indicated that tuftsin or tuftsin-like peptides exert multiple stimulatory effects on a subset of immunologic effector cells, including enhanced migration/chemotaxis, enhanced phagocyte respiratory burst, enhanced antigen presentation, and other undefined immunologic effects that result in increased antimicrobial and antitumor activities by immune cells (see the review by Siemion and Kluczyk (2)). Additionally tuftsin is reported to have effects on the nervous system, including induction of analgesia (3) and inhibition of axonal regeneration (2, 4).

Numerous proteins and peptides contain within them tuftsin or tuftsin-like sequences (2). Some of these, such as C-reactive protein, are known mediators of inflammation (5). In addition, two other proinflammatory peptides, substance P and neurotensin, both contain tuftsin-like sequences, stimulate phagocytosis, and can compete with [3H]tuftsin for binding to macrophages (6). Moreover fragments of substance P and neurotensin containing the tuftsin-like sequences maintain their tuftsin-like activity even though their interactions with the known substance P and neurotensin receptors are greatly reduced (6). This suggests that the tuftsin-like activities of those peptides are mediated by one or more additional receptors. Although a number of tuftsin-like peptides reportedly share the biological activity of tuftsin, several similar peptides, such as TKPPR, are potent tuftsin antagonists (2), indicating that there are highly specific structural requirements for tuftsin agonists.

Despite its 30-year history, the binding target(s) and mechanism of action of tuftsin remain largely a mystery. A definitive tuftsin receptor has yet to be cloned, although some have proposed that tuftsin binds to and activates a low affinity substance P or neurotensin receptor on macrophages (7). There are no reports, however, of a cloned receptor with a binding preference for the substance P and neurotensin fragments containing tuftsin-like sequences. Given the broad range of action of tuftsin in both the immune and nervous systems, one would expect the tuftsin receptor(s) to be common to both systems.

We unexpectedly found that cultured human aortic and umbilical vein endothelial cells possess tuftsin receptors and that in these cells the binding target for tuftsin is neuropilin-1. Because neuropilin-1 plays a critical role in the immune, vascular, and nervous systems and interacts with a number of different ligands, cell surface receptors, adhesion proteins, and intracellular proteins (8–10), we propose that at least some of the previously reported effects of tuftsin are mediated through neuropilin-1.

EXPERIMENTAL PROCEDURES

Materials—Red carboxylate and green fluorescent beads and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride (EDAC)2 were purchased from Molecular Probes. The peptide TKPPR was purchased from Bachem AG. Bovine serum albumin (BSA, Fraction V), glycine, MES, and Tween 20 were purchased from Sigma. Phospholipids 1,2-distearyl-sn-glycero-3-phosphatidylcholine (DSPC), 1,2-dipalmitoyl-sn-glycero-3-phospho-[γ32P]inositol, sodium salt (DPPGNa) were purchased from Lipoid. Polyethylene glycol 4000 was purchased from Omya. Palmitic acid and methylene chloride were obtained from Fluka. Perfluorobutane was purchased from F2 Chemicals. Dulbecco’s phosphate-buffered saline (D-PBS) was purchased from Invitrogen. Human aortic endothelial cells (HAECl) and human umbilical vein endothelial cells (HUVEC) were kindly provided by Dr. Myung Roh, University of Pennsylvania.

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2 The abbreviations used are: EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; BSA, bovine serum albumin; MES, 2-(N-morpholino)ethanesulfonic acid; DSPC, 1,2-distearyl-sn-glycero-3-phosphatidylcholine; DPPG/Na, 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol, sodium salt; D-PBS, Dulbecco’s phosphate-buffered saline; HAECl, human aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; NP-1, neuropilin-1; Fc, the Fc domain of human immunoglobulin; NP-2, neuropilin-2; VEGFR, vascular endothelial growth factor receptor; HATU/DIEA, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate diisopropylchloramine; DPPE, dipalmitoyl-sn-glycero-3-phosphoethanolamine; VEGF, vascular endothelial growth factor.
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(HUVEC) were obtained from Clonetics (Cambrex) and cultured in EGM-MV medium, also from Clonetics, on collagen-1-coated plasticware from BD Biosciences as indicated. MDA-MB-231 cells were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Invitrogen). A293H cells were obtained from Invitrogen and cultured in Dulbecco’s modified Eagle’s medium (high glucose) with 10% fetal bovine serum and 1× non-essential amino acids (Invitrogen). Radioimmunoprecipitation assay buffer was purchased from Teknova. 125I-VEGF165 (average specific activity about 1,500 Ci/mmol) was from Amersham Biosciences. Anti-VEGFR-2 antibodies (sc-504 and sc-315) were from Santa Cruz Biotechnology. Anti-neuropilin-1 (NP-1) antibody was from Santa Cruz Biotechnology (sc-7239). Anti-neuropilin-2 (NP-2) was from R&D Systems (MAB2215). Anti-β-tubulin (tub2.1) was from Sigma. Anti-VEGFR-1 antibody was from Calbiochem. Recombinant fusion proteins NP-1/Fc, NP-2/Fc, VEGFR-1/Fc, and VEGFR-2/Fc were from R&D Systems.

Preparation of Peptide-conjugated Beads—TKPPR was attached to red fluorescent carboxylate-modified beads (2.0-μm diameter) provided at 3.9 × 10^10 particles/ml. Fluorescent green unconjugated beads were used without modification as a control in experiments with cells. Peptide (1.0 mg) was combined with 0.5 ml of 50 mM MES buffer (Sigma), pH 6.0, and 0.2 ml of beads in a 1.5-ml microcentrifuge tube and rotated for 30 min at room temperature. Then 2.8 mg of EDAC in 0.025 ml of MES was added, and the tube was rotated for 2 h at room temperature. Following the addition of 0.005 ml of 1 N NaOH and 5.7 mg of glycine in 0.025 ml of MES, the tube was rotated for 30 min more at room temperature. The beads were then washed three times and resuspended in D-PBS.

TKPPR-conjugated Bead Binding Experiments—Red fluorescent beads derivatized with TKPPR were diluted with EBM medium (Bio-Whittaker) supplemented with 0.1% (w/v) BSA and aprotinin. Final bead concentration was 1.95 × 10^7/ml. Unconjugated green fluorescent beads were diluted with EBM/BSA buffer to give an equal bead concentration. Before starting the assay, bead suspensions were disaggregated in a sonication bath for 15 min. The wells of an 8-well chamber slide of confluent HAEC were drained of medium and rinsed with 0.5 ml/well EBM/BSA buffer. To each well 250 μl of bead solution (containing 4.9 × 10^6 beads) was added. The slide was incubated for 30 min on an orbital shaker at room temperature, drained, then washed once with 0.5 ml/well EBM/BSA buffer and twice with 0.5 ml/well D-PBS containing 2 mM MgCl2. Slides were mounted with Gel/Mount (Biomed) or Vectashield (Vector) aqueous mounting medium. Bead binding and localization was assessed at 40–300× magnification with an Olympus IMT-2 microscope equipped with a mercury lamp (Chiu Technical Corp., Model M-100) for fluorescence detection. Digital images were collected using a dual fluorescein isothiocyanate/Texas Red filter set.

Free TKPPR Peptide Inhibition of TKPPR-conjugated Bead Binding to HAEC—Binding to HAEC was carried out as above except that BSA was omitted during binding, and increasing concentrations of free TKPPR peptide were added to six of the eight wells. The binding period was reduced to 15 min, and 0.1% Tween 20 was present in the wash buffer. Digital images were collected of three different random fields in each well at 200× magnification with the fluorescent microscope setup described above to simultaneously detect red and green fluorescence. The images were segregated into separate red or green channels in Adobe Photoshop (image processing software, version 5.0), flattened (layer information removed), and saved as individual TIFF files. Micrografx Picture Publisher (version 7) was then used to enhance contrast by 100%. Finally the processed images were inverted into black on a white background using Scion Image software (version beta 3b), and the integrated density was measured with the whole field selected. The three images for each level of TKPPR competition and the six images of the control (from the two wells lacking competition from free peptide) were averaged and the percent inhibition of binding was calculated as % Inhibition = 100 × (Control Density – Competition Density)/Control Density. Where indicated, the percent inhibition was plotted against competitor concentration and curve-fitted using non-linear regression to the equation Y = (Bmax × X)/(Kd + X) with Prism software (version 3, GraphPad Inc.).

Synthesis of Phospholipid-TKPPR Conjugate—The fully protected peptide Gly-Thr(0Bzl)-Lys(Z)Pro-Pro-Arg(NO3)OBzl (where Z is benzoxycarbonyl and OBLz is benzoyloxy) was prepared in solution phase by t-butoxycarbonyl chemistry using HATU/DIEA as the coupling agent and methylene chloride as the solvent. The peptide was then treated with glacial anhydride, and the resulting monoacid was coupled with dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) in the presence of HATU/DIEA. After thoroughly purifying the compound, all the protecting groups on the product were removed by hydrogenation. The DPPE-glutaryl-GTKPPR was collected by precipitation with ether and characterized by mass spectrometry.

Formulation of TKPPR Microbubbles—A mixture of DSPC, DPPG-Na, palmitic acid, and 2% (mol/mol) phospholipid-TKPPR conjugate were dissolved together with polyethylene glycol 4000 (59 g/g of lipids) in tert-butyl alcohol. Vials were filled with 1.5 ml of solution, then frozen, and lyophilized. The air in the headspace was replaced with C2F10/N2 (35:65) mixture, and the vials were capped and crimped. Control formulations containing no TKPPR derivative were prepared using the same method.

Synthesis of TKPPR Multimers—Multimeric TKPPR constructs tagged with Oregon Green fluorescent marker were prepared using solution phase chemistry as described previously (11).

TKPPR-Microbubble Binding—Lyophilizates containing DPPE-glutaryl-TKPPR or control formulations were reconstituted with 5 ml of sterile saline using two needles (one for injection and one for venting to avoid overpressure) and agitated until complete dissolution of the cake. The microbubble suspensions obtained in this way were used within an hour of the assay. Microbubble solutions were diluted with D-PBS containing 0.2% BSA with or without added potential binding inhibitors as specified under “Results.”

Chamber slides containing confluent HAEC were drained of culture medium, which was immediately replaced with one of the final microbubble suspensions prepared above. The wells were sealed with a piece of Parafilm, inverted to allow the bubbles to rise and make contact with the cells, and incubated for 20 min after which the solutions were poured off. Weakly associated bubbles were removed by washing each well twice with 0.5 ml of D-PBS, swirling gently each time before pouring off. Additional D-PBS (0.5 ml) was added to wells after the washes to keep the cells submerged until microscopic evaluation.

TKPPR-Microbubble Binding Quantitative Competition with Potential Binding Antagonists—Chamber slides (8-well) and 48-well microtiter plates were seeded with HAEC as above or A293H cells. Bubble binding assays were carried out on the chamber slides or 48-well plates as described above except that the final concentration of BSA was 0.1%. Three representative fields from each well were digitally photographed at 300× magnification, and then bound bubbles were either manually counted or counted using Image Pro Plus (version 4.1) software. The percent inhibition of binding was calculated, and curve-fitting was performed as in the bead binding assays (above).
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Cell Transfections—A293H cells (Invitrogen) were transfected with an expression vector containing the hVEGFR-2 cDNA (cloned into pcDNA6, Invitrogen) (12) or the human NP-1 cDNA (Origene number TC117726) using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Cells were used for binding assays 24 h after transfection.

Radioligand Binding Assays—125I-VEGF165 binding to HUVEC, transfected A293H cells, or MDA-MB-231 cells in 96-well plates was carried out as described by Bikiyalvi et al. (13). The 125I-VEGF165 concentration was 300 pM, and binding was carried out for 1.5 h (HUVEC) or 2 h (MDA-MB-231 cells) at 4 °C in the presence of increasing concentrations of potential binding antagonists. Binding to recombinant VEGFR-2/Fc and NP-1/Fc was measured by coating high binding Maxi-Sorp 96-well plates (Nunc) with the receptor of interest at 3 μg/ml in D-PBS overnight at 4 °C. Plates were blocked with D-PBS with 1% BSA and 0.05% Tween 20 for 2.5 h and then washed with D-PBS with 0.1% Tween 20 prior to a 2-h incubation with the radioligand, held constant at 250 pM, at 4 °C following the same protocol as that used above to measure binding to cells. Competitive binding curves and IC50 values for one-site and two-site binding models were generated with Prism software.

Fluorescence Polarization Binding Assays—Fluorescence polarization studies were carried out using the FPM-1 fluorescence polarization analyzer obtained from Jolley Consulting and Research or an Analyst AD from Molecular Devices. Briefly the fluorescent TKPPR tetramer was held constant at 5 nM while the concentration of NP-1/Fc or other potential binding protein was increased as indicated. Polarization was read as soon as all the reagents had been combined. All measurements using the FPM-1 were made at 37 °C, whereas those made using the Analyst AD were performed at room temperature. Polarization in molar polarization units was plotted versus protein concentration, and KD values were calculated using non-linear regression and the equation Y = (Bmax × X)/(Kd + X) using Prism software. For competition studies, both the fluorescent tetramer and NP-1/Fc were held constant while the concentration of competitor was increased.

VEGFR-2 Activation Assays—Activation of VEGFR-2 was measured by immunoblot analysis of cell extracts from serum-starved HUVEC with or without stimulation with VEGF165 (5 ng/ml for 5 min) in the presence or absence of tetrameric TKPPR at 1 μM. Briefly VEGFR-2 was immunoprecipitated from lysates (lysate buffer = 20 mM Tris base, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 100 mM NaF, 50 mM sodium pyrophosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin) using anti-VEGFR-2 (sc-504), resolved by SDS-PAGE on a 7.5% gel, electroblotted onto a polyvinylidene difluoride membrane, probed with an anti-phosphotyrosine antibody (PY-20), stripped, and reprobed with another anti-VEGFR-2 antibody (sc-315). Primary antibody binding was visualized by probing with a horseradish peroxidase-conjugated secondary antibody, applying ECL chemiluminescent substrate (Amersham Biosciences), and exposing to X-Omat film (Eastman Kodak Co.).

Protein Expression by Immunoblot Analysis—Cell extracts prepared in radioimmune precipitation assay buffer were resolved by SDS-PAGE, electroblotted onto polyvinylidene difluoride membranes, probed with anti-NP-1 and anti-NP-2 antibodies, stripped, and reprobed with an anti-β-tubulin antibody to control for protein loading. Primary antibody binding was visualized by probing with a horseradish peroxidase-conjugated secondary antibody followed by chemiluminescence detection as above.

RESULTS

Binding a Tuftsin Antagonist to Endothelial Cells—During an effort to identify ligands capable of targeting radioisotopes and lipid-based microbubbles to diseased vascular sites for use in diagnostic imaging, we measured the binding of fluorescent beads (average 2-μm diameter) with TKPPR conjugated to their surface to cultured HAEC. We used fluorescent beads in the assay as surrogates for microbubbles because they were more amenable to ligand attachment. In comparison with unconjugated beads, which showed little affinity for HAEC, TKPPR-conjugated beads bound well to endothelial cells (Fig. 1A). This binding was almost entirely restricted to cell surfaces, and in areas where cells had come off the slide, few TKPPR beads bound to the underlying matrix (not shown). We quantified the binding of fluorescent beads to endothelial cells by fluorescence microscopy and found that free TKPPR peptide blocked most bead binding with an IC50 of about 14 μM (Fig. 1B), indicating that the binding was due to a specific interaction with tuftsin receptors on endothelial cells. About 25% of bead binding could not be blocked with free TKPPR possibly due to mechanical trapping.

Several reports suggest that cultured endothelial cells are less quiescent than normal endothelial cells in vivo, displaying some of the traits of endothelial cells involved in wound healing or angiogenesis (14, 15). Because the presence of tuftsin receptors had previously been described mainly associated with inflammation, we hypothesized that the presence of tuftsin receptors on cultured endothelial cells was reflecting their inflammatory phenotype. This suggested that ultrasound bubbles targeted to tuftsin receptors may be useful for imaging vascular inflammation in vivo. Therefore we prepared ultrasound bubbles conjugated with the TKPPR sequence on their surface. When we added a TKPPR phospholipid to a formulation of lipids to generate ultrasound bubbles (see “Experimental Procedures”) the resulting bubbles gained the ability to adhere to endothelial cells (Fig. 1C). Bubbles prepared with the same lipid formulation lacking the TKPPR phospholipid did not bind HAEC under the same conditions. TKPPR bubble binding to endothelial cells was specific as evidenced by the ability of free TKPPR peptide to inhibit the binding with an IC50 of about 4.5 μM (Fig. 1D). The bubbles showed very little binding to denuded sites within the monolayer of cells, and when tested, bubble binding to HUVEC was comparable to the binding to HAEC.

The Tuftsin Receptor on Endothelial Cells Is a Neuropilin—To characterize the binding of TKPPR bubbles to endothelial cells we tested a variety of substances for binding inhibition. The results of these studies are summarized in Table 1. The native ligand, TKPR (tuftsin), was more than 20-fold less potent than free TKPPR peptide, consistent with reports that TKPPR has a greater affinity for the tuftsin receptor (2). A tetrameric form of TKPPR (Fig. 2A) was 45-fold more potent than monomeric TKPPR.

The lack of inhibition by the basic peptide RRRRR (Table 1) indicated that TKPR binding to endothelial cells was not merely charge-related. To the contrary, a cyclized version of TKPR, CTKPPPRC, lost all inhibitory activity suggesting that binding to the tuftsin receptor requires a specific conformation.

Based on the observation that VEGF ends in a tuftsin-like sequence, KPRR, we tested VEGF165 in a bubble binding assay and observed that it potently blocked TKPPR-conjugated bubble binding to endothelial cells with an IC50 of 0.3 nM. VEGF165 binds specifically to VEGFR-1, VEGFR-2, NP-1, and NP-2 as well as nonspecifically to cell surface and extracellular matrix proteoglycans (16). The latter interaction, as well as binding to NP-1 and NP-2, is mediated through the heparin-binding domain of VEGF. We reasoned that if VEGF165 were competing through its heparin-binding domain the competition should be revers-
ible by adding heparin. Consistent with this, combining 1 mg/ml heparin with VEGF almost completely restored bubble binding to non-VEGF treatment levels (Table 1). Heparin alone in the absence of VEGF165 had no effect. These results suggest that VEGF165 and TKPPR compete for binding to NP-1, NP-2, or a cell surface proteoglycan. We discounted extracellular matrix as the binding target because TKPPR bubbles demonstrated very little affinity for the extracellular matrix exposed in denuded sites.

If TKPPR bubble binding is inhibited by the heparin-binding domain of VEGF, then VEGF121, which lacks that domain (16), should not prevent bubble binding. Consistent with this, we found that VEGF121 had no effect on TKPPR bubble binding to HUVEC (Table 1). Placental growth factor-1, a VEGF-related proangiogenic factor that binds to VEGFR-1 but not NP-1 (16), also had no effect at the highest concentration tested (27 nM). To determine whether TKPPR binds to neuropilin-1 rather than a cell surface proteoglycan, we tested recombinant NP-1/Fc chimera for its ability to block bubble binding to HUVEC. We found that NP-1/Fc potently blocked bubble binding with an IC50 of about 7 nM indicating that NP-1 is at least one of the bubble binding targets. TKPPR bubble binding to neuropilin-1 was also confirmed by demonstrating direct binding to an NP-1/Fc-coated 96-well plate. This binding could be inhibited by free TKPPR peptide and did not occur in uncoated wells or wells coated with VEGFR-2/Fc. As a further control, unconjugated bubbles were tested and found not to bind to wells coated with NP-1/Fc.

**TKPPR Blocks VEGF165 Binding to Neuropilin-1**—If VEGF165 can block TKPPR-conjugated bubbles from binding to neuropilin-1 one would expect TKPPR to inhibit labeled VEGF165 binding to NP-1 or NP-1-expressing cells. To test this we added increasing concentrations of TKPPR or dimeric or tetrameric versions of TKPPR (Fig. 2 A) during incubation of 125I-VEGF165 in 96-well plates coated with NP-1/Fc or VEGFR-2/Fc. As shown in Fig. 2B and Table 2, TKPPR and its dimeric and tetrameric analogs competed with 125I-VEGF165 for binding to NP-1/Fc with IC50 values of 46, 7.9, and 0.5 μM, respectively, by nonlinear regression. The shallowness of the inhibition curve generated with tetrameric TKPPR suggested that the data might better fit a two-binding site model rather than a one-site model. When the assay was repeated with additional data points to allow two-site fitting, this proved to be the case (Fig. 2C). The goodness of fit (R2 value) was 0.9999 for the two-site model as opposed to 0.9912 for the one-site model, indicating that the two-site model was a better fit through the data points. The IC50 values generated for the two sites were 0.1 and 3.5 μM. TKPPR had no effect on 125I-VEGF165 binding to VEGFR-2/Fc at up to 100 μM (not shown).

**TABLE 1**

| Substance | IC50  |
|-----------|-------|
| TKPR      | 100 μM|
| TKPPR     | 4.5 μM|
| CTKPPRC (200 μM) | No inhibition |
| RRRRR (90 μM) | No inhibition |
| (TKPPR)4 | 0.1 μM|
| VEGF165  | 0.0003 μM|
| VEGF165 (14.3 nM) | No inhibition |
| PI GF-1 (27 nM) | No inhibition |
| IL-1α (59 nM) | No inhibition |
| IL-1α (59 nM) | No inhibition |
| Heparin (1 mg/ml) | No inhibition |
| VEGF165 (1.2 μM) | 93% inhibition |
| VEGF165 (1.2 μM) + heparin (1 mg/ml) | 18% inhibition |
| NP-1/Fc  | 0.007 μM|

α Placental growth factor-1.  
β Interleukin-1α.
On HUVEC, tetrameric TKPPR inhibited $^{125}$I-VEGF$_{165}$ binding only partially, achieving most of its maximum inhibition (40–60%) around 2 $\mu$M (Fig. 2D). Half of the maximal inhibition was reached around 0.1 $\mu$M, similar to the IC$_{50}$ for tetrameric TKPPR on NP-1/Fc-coated plates. Because only about half or fewer of the VEGF receptors on HUVEC are NP-1 (17), these results are consistent with selective competition by tetrameric TKPPR for binding to NP-1 on these cells. On A293H cells transfected with VEGFR-2, tetrameric TKPPR had no effect on $^{125}$I-VEGF$_{165}$ binding (not shown), confirming that the TKPPR sequence interacts with a neuropilin but not VEGFR-2.

MDA-MB-231 is a breast carcinoma cell line that has been reported to express NP-1 as its sole VEGF receptor (17–19). We found that tetrameric TKPPR was able to potently block almost 100% of $^{125}$I-VEGF$_{165}$ binding to these cells (Fig. 2E). Again the data fit a two-binding site model best, yielding IC$_{50}$ values of 40.4 nM and 2.27 $\mu$M. However, other...
groups have reported NP-2 protein and VEGFR-1 and VEGFR-2 mRNA expression in these cells (for a review by Barr et al., see Ref. 20). We analyzed lysates from MDA-MB-231 cells for these proteins by immunoblot analysis. We found, in agreement with previous reports (17, 19), that NP-1 was more abundant in MDA-MB-231 cells than in HUVEC (Fig. 3A). VEGFR-1 and especially NP-2, however, were expressed at much lower levels in MDA-MB-231 cells than in HUVEC (Fig. 3, B and C). VEGFR-2 protein was not detected in the MDA-MB-231 cells. Based on these results, we conclude that most VEGF binding to the MDA-MB-231 cells used in our study was mediated by NP-1, consistent with the ability of tetrameric TKPPR to almost fully block VEGF_{165} binding to these cells.

Neuropilin-1 and neuropilin-2 are believed to stimulate angiogenesis by acting as co-receptors for VEGFR-2 whereby they enhance the binding of certain VEGF isomers to VEGFR-2 and/or the signal transduction that occurs after VEGF binding to VEGFR-2 (16, 17). It is believed for this reason that isoforms that do not bind to the neuropilins, such as VEGF_{121}, are less potent than those that do (16). Agents that block the binding of VEGF_{165} to NP-1/NP-2 have been shown to reduce (but not completely prevent) the activation of VEGFR-2 by VEGF as reflected in the presence of increasing concentrations of NP-1/Fc, VEGFR-1/Fc, VEGF_{2}/Fc, VEGF_{165} and NP-2/Fc. Only NP-1 was able to significantly increase the polarization from the fluorescent tetramer with a $K_D$ of 23, 38, or 53 nM (average, 38 nM) depending on the instrument and assay protocol used (Fig. 4, A, B, and C; see “Experimental Procedures” for assay details). When a fluorescently labeled TKPPR monomer was used in the assay, no polarization was measurable at up to 100 nM NP-1/Fc (not shown). This is consistent with the much lower potency (4.5 or 46 μM) observed for monomeric TKPPR in blocking TKPPR-bubble and VEGF_{165} binding to endothelial cells.

VEGFR-2/Fc and VEGF_{165} did not bind the TKPPR tetramer at all at up to 80 and 250 nM (Fig. 4, A and C), whereas NP-2/Fc appeared to produce only a weak polarization from the tetramer at about 120 nM (Fig. 4B). VEGFR-1/Fc produced a weak polarization of the TKPPR tetramer that was non-saturable through 250 nM, suggesting either non-specific or very low affinity binding. The binding of the tetramer to NP-1 in this assay could be completely blocked by free TKPPR and VEGF_{165}, whereas NP-2/Fc and VEGF_{121} did not bind VEGF_{165}. This results along with the inability of TKPPR to block $^{125}$I-VEGF_{165} binding to VEGFR-2-transfected and NP-2/Fc-transfected A293H cells suggest that selective binding of TKPPR to NP-1, although a weaker interaction with NP-2 and VEGFR-1 cannot be completely ruled out. The fact that VEGF_{165} was able to fully block fluorescent TKPPR tetramer binding to NP-1 and did not generate any polarization from the tetramer directly (Fig. 4C) excludes the possibility that the TKPPR tetramer works by binding the NP-1-binding site on VEGF such that it can no longer bind neuropilin-1.

**TABLE 2**

| Substance          | IC_{50} \( \mu M \) |
|--------------------|----------------------|
| TKPPR              | 46                   |
| (TKPPR)$_2$        | 7.9                  |
| (TKPPR)$_4$, one-site model | 0.5                |
| (TKPPR)$_4$, two-site model, high affinity | 0.1                |
| (TKPPR)$_4$, two-site model, low affinity | 3.5                |
| VEGF_{165}         | 0.001                |

**FIGURE 3.** Immunoblot analysis of VEGF receptor expression and VEGFR-2 activation. A–C, immunoblot analysis of the indicated VEGF receptors in lysates of HUVEC (leftmost lane) and MDA-MB-231 cells (rightmost lane). The lower panels show the results of stripping each blot and reprobing for β-tubulin. D, inhibition of VEGF-induced VEGFR-2 autophosphorylation by TKPPR tetramer.
the TKPPR bubbles bound to the NP-1 but not mock-transfected cells, these data indicate that TKPPR at least has a higher affinity for NP-1 over NP-2 in agreement with the fluorescence polarization data.

**DISCUSSION**

We have found that the tuftsin antagonist TKPPR binds to neuropilin-1. In so doing it prevents VEGF from binding to neuropilin-1 and thereby reduces VEGFR-2 activation by VEGF. Others (22) have also observed that selectively blocking VEGF-neuropilin-1 interaction using fragments of exon 7 (sometimes combined with exon 8) from VEGF significantly reduces VEGF signaling through VEGFR-2. This is consistent with the proposed role of neuropilin-1 as a VEGF co-receptor (16, 21, 22).

Exon 8 at the 3' terminus of the VEGF-A gene codes for a short sequence with homology to tuftsin and TKPPR. This exon is highly conserved, but a function for the six amino acids it codes for has not been proposed previously. VEGF<sub>110</sub>, a proteolytic fragment of VEGF<sub>121</sub>, lacks both exons 7 and 8 yet retains VEGF-2 binding and angiogenic activity although at a reduced level (23). We suggest that the KPRR sequence of exon 8 binds to neuropilin-1 separately from the heparin-binding domain coded for by exon 7 and helps to orient VEGF for optimal binding to VEGFR-2. Consistent with this, a VEGF-like protein coded for by the NZ2 strain of the orf virus, ORFV2-VEGF, ends in the sequence TRPPRRR and binds to NP-1 in addition to VEGFR-2 even though it lacks a heparin-binding domain (24). A recently described splice variant of VEGF, VEGF<sub>165b</sub>, only differs from VEGF<sub>165</sub> in having the six amino acids of exon 8 replaced with SLTRKD (25). VEGF<sub>165b</sub> has no angiogenic activity and antagonizes the effects of VEGF<sub>165</sub> in cell-based assays (25), suggesting that when VEGF interacts with neuropilin-1 exon 8 plays an important role.

VEGF<sub>121</sub> ends in the six amino acids coded for by exon 8 but reportedly does not bind to neuropilin-1 (16, 17). We suggest that VEGF<sub>121</sub> may have modest affinity for neuropilin-1 that is not easily detected by traditional methods. When bound to VEGFR-2, the KPRR motif of VEGF<sub>121</sub> may interact with neuropilin-1 to enhance the binding to a second VEGFR-2 molecule and/or adjust the conformation of the two bound VEGFR-2 molecules to enhance signaling. This is supported by experiments showing that a recombinant fragment of VEGF comprising
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FIGURE 5. TKPPR-conjugated bubble binding to A293H cells under various transfection conditions. Scale bar = 30 μm. A, binding to control untransfected cells. B, binding to mock-transfected cells. C, binding to VEGF2-transfected cells. D, binding to NP-1-transfected cells. E, binding to NP-1-transfected cells in the presence of 1 μM free TKPPR tetramer peptide. F, immunoblot analysis of 25 μg of NP-1-transfected (lane 1) and 50 μg of mock-transfected (lane 3) A293H cell lysates for NP-1 and NP-2 expression. Lane 2 was left blank. The lower panels show the results of stripping each blot and reprobing for β-tubulin.

the amino acids coded for by exons 7 and 8 was able to partially inhibit the binding and biological activity of VEGF121 when added to cultured endothelial cells even though VEGF121 lacks the peptide sequence coded for by exon 7 (19).

Until now, due to the lack of a cloned or purified active receptor, the tuftsin receptor has simply been defined as whatever tuftsin binds to, limiting efforts to determine its mechanism of action. Our results indicate that neuropilin-1 is a tuftsin receptor although not necessarily the only one. It remains to be determined which of the effects of tuftsin can be attributed to its interaction with neuropilin-1, but it is interesting to note that the stimulation or inhibition of neuropilin-1 function reproduces many of those effects including modulation of the immune system (8), axonal regeneration (9), and inflammation (26). Consistent with this, neuropilin-1 expression has been reported in many of the same cell types described as targets of tuftsin action, including immune cells (8, 27) and neurons (9, 16). Semaphorin 3 proteins, which play roles in the nervous system, immune system, and angiogenesis, compete with VEGF for binding to neuropilin-1. Although endothelial cells have not been reported to have tuftsin receptors, they have never been excluded from having them either. It should be pointed out also that angiogenesis is closely linked with inflammation, and therapies that inhibit or enhance angiogenesis can inhibit or enhance inflammation (29–31), so some tuftsin effects attributed to inflammation may have in fact involved endothelial cells and angiogenesis. Based on this, it is possible that the reported uptake of a radiolabeled TKPPR derivative by arthritic tissues in an imaging study by Caveillers et al. (32) reflected angiogenesis in those tissues in addition to, or in place of, inflammation.

Multimerization greatly increased the binding affinity of the TKPPR sequence for neuropilin-1, lowering the IC50 for competition with VEGF from about 46 μM for the monomer to 100 nM for the tetramer. Similarly the IC50 for inhibiting TKPPR bubble binding was decreased from 4.5 μM for monomeric TKPPR to 100 nM for the tetramer. The reported affinity of tuftsin for receptor-positive cells is in the 50–130 nM range without apparent multimerization (2); this raises the question: how can neuropilin-1 be a tuftsin receptor if its affinity for the ligand is so much lower than that reported? One potential explanation is that tuftsin becomes multimerized during the assay. Najjar and Bump (33) reported that [3H]Pro3]tuftsin became covalently attached to an acceptor protein on the surface of HL-60 and rabbit granulocyte cells. Tissue transglutaminase present on the surface of these cell types (34) can cross-link peptides to membrane proteins as well as soluble proteins (35), providing a plausible mechanism by which multimeric tuftsin could be generated.

We demonstrated that TKPPR on the surface of microbubbles allowed them to adhere to cultured endothelial cells and that this binding was at least in part mediated by neuropilin-1. Such bubbles may prove useful in imaging the angiogenesis and/or inflammation associated with a number of different pathologies. In addition, multivalent TKPPR may be useful in selectively delivering drugs, radioisotopes, and even gene therapy constructs to these sites. Because tumor cells themselves frequently overexpress NP-1 (16), TKPPR-based agents capable of diffusing out of blood vessels may be useful for imaging or treating malignancies through both antiangiogenic as well as direct tumor targeting mechanisms simultaneously.

In summary, we have shown that neuropilin-1 is a receptor for the immunomodulatory peptide tuftsin. Based on the ability of a multimeric tuftsin analog to reduce VEGF signaling through VEGFR-2 and the striking overlap in processes modulated by tuftsin and NP-1, we propose that at least some of the previously reported effects of tuftsin are mediated through neuropilin-1.
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