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UROKINASE-CONTROLLED TUMOR PENETRATING PEPTIDE

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Abstract

Tumor penetrating peptides contain a cryptic (R/K)XX(R/K) CendR element that must be C-terminally exposed to trigger neuropilin-1 (NRP-1) binding, cellular internalization and malignant tissue penetration. The specific proteases that are involved in processing of phage display identified tumor penetrating peptides are not known. Here we design de novo a tumor-penetrating peptide based on consensus cleavage motif of urokinase-type plasminogen activator (uPA). We expressed the peptide, uCendR (RPARSGR↓SAGGSVA, ↓ shows cleavage site), on phage or coated it onto silver nanoparticles and show that it is cleaved by uPA, and that the cleavage triggers binding to recombinant NRP-1 and to NPR-1-expressing cells. Upon systemic administration to mice bearing uPA-overexpressing breast tumors, FAM-labeled uCendR peptide and uCendR-coated nanoparticles preferentially accumulated in tumors tissue. We also show that uCendR phage internalization into cultured cancer cells and its penetration in explants of murine tumors and clinical tumor explants can be potentiated by combining the uCendR peptide with tumor-homing module, CRGDC. Our work demonstrates the feasibility of designing tumor-penetrating peptides that are activated by a specific tumor protease. As upregulation of protease expression is one of the hallmarks of cancer, and numerous tumor proteases have substrate specificities compatible with proteolytic unmasking of cryptic CendR motifs, the strategy

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described here may provide a generic approach for designing proteolytically-actuated peptides for tumor-penetrative payload delivery.

Graphical abstract

Keywords

C-end Rule; neuropilin-1; urokinase; α_v integrins; tumor targeting; silver nanoparticles

INTRODUCTION

Distinct molecular features of tumor vessels can be used for affinity-based (synaptic) delivery of diagnostic and therapeutic cargo using various affinity ligands (e.g. peptides, antibodies). A prototypic tumor-penetrating peptide, iRGD (CRGDKGPDC), combines synaptic targeting with tumor-specific vascular exit and tissue penetration [1]. The activity of iRGD depends on the cell -internalization and tissue-penetration sequence motif R/KXXR/K. We have termed this motif the C-end Rule or CendR motif and the endocytotic pathway it triggers the CendR pathway [1,2]. iRGD is a composite of the tumor-homing RGD motif, which binds to α_v -integrins in angiogenic tumor vessels, and an overlapping cryptic RGDK CendR motif. Proteolytic cleavage of iRGD to unmask the CendR motif in tumors [1] provides a key regulatory step, whereby iRGD loses affinity for integrins, while acquiring NRP-1-binding activity, which induces extravasation and cell and tumor penetration [1,3]. The identity of the iRGD processing protease(s) is not known. During tumorigenesis, extracellular proteolytic machinery is activated at a number of levels (transcriptional activation, increased secretion, upregulation of cell surface receptors for proteases, and downregulation of protease inhibitors) [4,5]. Of the ~600 proteases in the human degradome, many have substrate specificities compatible with C-terminal unmasking of R/KXXR/K required for CendR activation. We hypothesized that it may be possible to design cryptic CendR peptides that are activated by specific tumor proteases to trigger tumor penetration.

Urokinase-type plasminogen activator (uPA), proteolytically converts plasminogen to the broad spectrum protease, plasmin. In healthy adults, uPA generally occurs with low abundance and has a limited tissue distribution. uPA overexpression is firmly linked to angiogenesis, tumor invasion and metastasis [6,7]. In tumors, high-affinity uPA receptor

(uPAR) concentrates active uPA to the surface of tumor cells, macrophages, and angiogenic endothelial cells [8]. uPA and its receptor are key components of a cell surface proteolytic cascade used by both tumor cells and activated capillary endothelial cells for basement membrane invasion, a process required for metastasis and angiogenesis. In response to angiogenic agents, endothelial cells in tumor neovessels secrete uPA and upregulate cell surface expression of uPAR [6–8]. uPA has been used for protease-activated systemic drug targeting, and its use is further supported by successful application of uPA-mediated activation of anthrax toxin [9,10], cytotoxic proteins [11], and cytotoxic drug conjugates [12] for tumor therapy.

The aim of the current study was to design a urokinase-activatable tumor-penetrating peptide, which we call uCendR, and validate its activity for cell- and tumor penetrative delivery of payloads. Our study demonstrates the feasibility of rationally designing tumor-penetrating peptides that are activated by specific tumor proteases. These findings expand the versatility and utility of CendR-based platforms in tumor targeting.

MATERIALS AND METHODS

Peptides and proteins

Peptides were purchased from Lifetein Inc. (Somerset, NJ), or synthesized using Fmoc/t-Bu chemistry on a microwave assisted automated peptide synthesizer (Liberty, CEM Corporation, Matthews, NC). Peptides were purified by high-performance liquid chromatography (HPLC) using 0.1% TFA in acetonitrile-water mixtures to 90%–95% purity and validated by Quadrupole-time-of-flight (Q-TOF) mass spectral analysis. Fluorescent and biotinylated peptides were synthesized by using 5(6)-carboxyfluorescein (FAM) or biotin, each with 6-aminohexanoic acid (Ahx) spacer between it and the N-terminus of the peptide. NRP-1 b1b2 protein was purified at the Protein Production and Analysis Facility at the Sanford Burnham Prebys Medical Discovery Institute (La Jolla, CA).

Silver nanoparticle synthesis, functionalization

Silver nanoparticles (AgNPs) were prepared by citrate reduction as described [13]. An extinction of $1 \times 10^{10} \text{ M}^{-1} \text{ cm}^{-1}$ at the 405 nm Ag plasmon peak was used to quantify the concentration of AgNPs. The synthesis of Ag citrate (20–50 nm diameter) was performed as follows: AgNO_3 (450 mg, Sigma) was dissolved in 2.5 L water and vigorously boiled while stirring, and trisodium citrate dihydrate (500 mg, Sigma) in 50 mL water was added. After 20–30 min the solution became greenish brown, at which point the heat was turned off and the solution stirred overnight. UV-Vis showed an absorbance of ~ 10 at 405 nm, 1 cm pathlength. Neutravidin (NA) was conjugated to 5 kDa heterobifunctional succinimidyl carboxymethyl ester polyethylene glycol (PEG) orthopyridyl disulfide (NHS-PEG-OPSS, Jenkem Technology, Plano, TX) and dialyzed against $0.1 \times$ phosphate-buffered saline (PBS) as described [13]. The NA product (3.1 mg/mL, 2 mL, with ~ 2 PEG-OPSS per NA) was added to 500 mL Ag, sonicated, then 25 mL of 50 mM morpholino sulfonic acid (MES) buffer, pH 6 (adjusted with HNO_3) was added and incubated overnight at 37°C . The solution was cooled to room temperature and 50 mL of $10 \times$ PBS was added, final pH ~ 7.4 . Then 250 μL of Tween 20 (10 weight-%) in water was added to reduce adsorption of colloid to plastic.

Ag was centrifuged (Thermo Nalgene Cat #3140-0500, 500 mL) at 12k $\times$ g at 4°C 1 h, and redispersed (bath sonication, Branson model 1510), then combined and spun in a 40 mL tube at 17k $\times$ g 20 min 4°C. The pellet was redispersed in PBS with 0.005% Tween 20 (PBST). Next, lipoic acid PEG amine (LPN, 5.7 mg of 1000 g/mol, Nanocs, #PG2-AMLA-1k) was dissolved in 570 μ L 0.1 M tris(2-carboxyethyl)phosphine hydrochloride solution pH 7 (TCEP, Sigma) and allowed to reduce the lipoic acid group for at least 1 h. The Ag was treated with 1 mM TCEP for 30 min to reduce the disulfides of the NA-OPSS. Then LPN was added (0.05 mg/mL) and incubated at 37°C for 1 h. The product was pelleted and redispersed in PBST, filtered using 0.45 μ m syringe filter (PVDF, Millipore Cat# SLHV033RS), and stored at 4°C. Optical density was $\sim$ 300 at 1 cm, 405 nm Ag plasmon peak. For dye-labeling, 20 μ L of 2 mM amine-reactive NHS-Oregon Green 488 (Thermo, Cat#O-6147) dissolved in DMSO was added per mL of Ag, followed by 15 μ L 7.5% sodium bicarbonate solution (Gibco, Cat#25080). The labeling occurred at room temperature for 1 h then overnight at 4°C, then washed 3 $\times$ (7k $\times$ g, room temperature), re-dispersing in PBST. The biotinylated peptides were loaded into the AgNPs, washed by centrifugation and brought up in PBST. Prior to use, Ag was filtered (0.22 μ m, Millipore Cat#SLGV013SL).

***In vitro* cell uptake with silver**

PPC-1 or M21 cells were seeded into 96 well plates and used the following day. Peptide loaded AgNPs (25 μ L, 200 O.D.) were incubated in eppendorf tubes with or without 5 μ L of uPA (Calbiochem #672112, stock 10 000 u/mL) for at least 30 min at 37°C, then 3 μ L was added to cells for 90 min at 37°C. For etching, 5 μ L of etchant (20 mM K₃Fe(III)(CN)₆ and 20 mM Na₂S₂O₃ in PBS) was added prior to imaging. Imaging was performed as described [13] under dark field illumination to visualize the AgNP core (20 $\times$ objective, Leica DMIRE2).

Fluorescence assay with silver

Peptide loaded AgNPs (25 μ L, 300 O.D.) were incubated in eppendorf tubes with or without 5 μ L of uPA (Calbiochem #672112, stock 10 000 u/ml) or 5 μ L PBS for at least 30 min at 37°C. Note that for biotin-blocked AgNPs, excess D-biotin in DMSO was added 15 min prior to adding the biotin-Ahx-GGSGRPARSGRSAGGK(Rho)DA-OH. Then, 5 μ L AgNP solution was diluted into 200 μ L PBS in 96 well plates (black wall, clear bottom) and read on a Tecan plate reader. AgNPs without OR488 label or peptide was used as a background control, and gave negligible signal above blank PBS. Assay conditions were OR488 channel: excitation 485 nm, emission 538 nm, cutoff 515 nm; Rho channel: excitation 544, emission 590, cutoff 570.

Quantum dots

Streptavidin ITK-605 quantum dots (Invitrogen, Carlsbad, CA) were modified with biotinylated peptides by incubation with a 100-fold molar excess of peptide followed by removal of free peptide by dialysis.

Mice and cell lines

All animal experimentation was performed according to procedures approved by the Animal Research Committee at the University of Sanford Burnham Prebys Medical Discovery Institute (La Jolla, CA). For tumor injections and before sacrificing, the mice were anesthetized with intraperitoneal injections of xylazine (10 mg/kg) and ketamine (50 mg/kg). BALB/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were used for tumor xenografts. For histological analysis, tissues were fixed in 4% paraformaldehyde, cryoprotected in phosphate buffered saline solution containing 30% sucrose, frozen, and sectioned at 10µm. PPC-1, 4T1, and M21 cell lines were maintained in the Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin.

Peptide phage display

T7-select phage display system (EMD Biosciences, Gibbstown, NJ) was used for individual peptide-phage cloning according the manufacturer's instructions. Phage was purified by precipitation with PEG-8000 (Sigma, St. Louis, MO) followed by CsCl₂ gradient ultracentrifugation and dialysis. The sequences of displayed peptides were determined from the DNA encoding the insert-containing region at the C-terminus of the T7 major coat protein gp10.

For phage binding studies [14], cultured cells were grown to near-confluence and dissociated with trypsin, and mouse organs were dissociated using the Medimachine system (BD Biosciences, San Jose, CA). To measure phage binding, 10⁶ cells in binding buffer (DMEM containing 1% bovine serum albumin, BSA) were incubated with 10⁹ pfu/ml of T7 phage for 1 hour at 4°C. The cells were washed 4 times with the binding buffer, lysed in LB bacterial growth medium containing 1% NP-40, and phage was titrated. Phage internalization assays used the same procedure, except that the cells were incubated with the phage at 37°C. For *in vitro* phage binding, phage was incubated with recombinant hexahistidine-tagged NRP-1 b1b2 coated on Ni-NTA magnetic beads (Qiagen, Hilden, Germany) in washing buffer (PBS containing 0.05% Tween-20, 5 mM imidazole and 0.5% BSA, pH 7.4). Phage particles (10⁸) were incubated in 0.5 mL of the buffer for 1 h followed by 4 washes and elution of protein-phage complexes in PBS containing 400 mM imidazole. Eluted phage was quantified using plaque assay.

Protease treatment of phage and nanoparticles

10⁹ phage particles or peptide-coated nanoparticles (volume 50 µl) were treated with uPA (Calbiochem #672112, stock 10 000 u/mL), or with other proteases: 25 µg of crystalline trypsin, 50 iu of thrombin, or 25 µg of collagenase type I (all Sigma, St. Louis, Mo) at 37°C for 30 min, followed by cell binding at 4°C for 3 hrs.

In vivo peptide and nanoparticle homing

Synthetic peptides labeled with fluorescein (200 µg) were intravenously injected into tumor-bearing mice and allowed to circulate for 15 min to 2 h. The mice were perfused through the heart with PBS containing 1% BSA, the tissues were collected and observed under blue light

(Illumatool Bright Light System LT-9900, 470/515 nm excitation/emission), and then processed for immunofluorescence or immunohistochemistry.

For AgNP homing, peptide loaded materials (100 μ L, 200 O.D. in PBST) were injected via tail vein, allowed to circulate for 4 h, then mice were perfused through the heart with 30 mL etchant solution (1 mM $K_3Fe(III)(CN)_6$ and 3 mM $Na_2S_2O_3$ in PBS). Tissues were removed and fixed, cryoprotected, frozen in O.C.T compound (Tissue-Tek) and 10 μ m sections were collected on glass slides.

Autometallographic staining was performed as previously described [13]. Briefly, tissue sections were air dried, rehydrated in PBS, washed with water, then blocked with pre-stain solution for 10–20 min (glycine 50 mM pH 7.8, 2% trisodium citrate dihydrate, Triton-X100 0.4%, 0.04% sodium azide), and washed with water. Slides were loaded into a Sequenza slide rack system (Ted Pella, Cat# 36105&36107), and washed with 2 mL water, then autometallographically stained using the LI Silver Enhancement kit (LifeTech Cat#L-24919) for 40–45 min, adding freshly prepared growth solution every 15 min. The reaction was quenched using 0.1 M $Na_2S_2O_3$ in water, then slides were washed with water, transferred to a coplin jar, and dehydrated through a series of ethanol in water until reaching pure ethanol. Slides were cleared in xylene and mounted in DPX (refractive index 1.52, Sigma Cat#06522) with glass coverslip. Dark field imaging was performed on the tissues using a 10 $\times$ objective on a Leica DMIRE2, with 4 regions per tissue analyzed in ImageJ software using consistent thresholding for quantification of percent area of positive pixels, which indicates silver deposits.

Ex vivo tumor penetration assay (tumor dipping assay)

Tumors (~0.8 cm diameter) were excised and incubated in DMEM containing 10^9 pfu/ml of T7 phage for 90 min at 37°C. After the incubation, tumors were washed with cold DMEM containing 1% BSA, fixed, sectioned, immunofluorescently stained, and viewed with a confocal microscope. Fresh surgical samples of peritoneal metastases of appendiceal cancer were collected under protocols approved by an Institutional Review Board at the University of California San Diego.

Immunofluorescence staining

2×10^5 cells were grown in 6-well tissue culture plates on collagen-I-coated coverslips (BD Biosciences, San Jose, CA) overnight at 37°C in 5% CO_2 , and incubated with 10^8 pfu of T7 phage. The cells were fixed in 4% paraformaldehyde or cold (–20°C) methanol, and stained with antibodies. Cell nuclei were stained with DAPI or Hoechst 33342. Primary antibodies used were rat monoclonal anti-mouse CD31 antibody (BD Biosciences), rabbit anti-NRP-1, mouse anti-NRP-1 (Miltenyi Biotec Inc., Auburn, CA). The secondary antibodies, Alexa594 goat antibodies to mouse, rat, and rabbit immunoglobulins and Alexa488 donkey anti-rabbit antibody were from Invitrogen (Carlsbad, CA). Cells and tissue sections were examined by confocal microscopy (Fluoview 500, Olympus America Inc., Center Valley, PA).

In situ zymography of plasminogen activators

Ten- μ m cryosections on microscope slides were brought at room temperature; overlaid with a solution containing dry milk, plasminogen, and agar, and incubated in a humid chamber at 37°C [15]. To distinguish between the two PAs, a specific inhibitor of uPA, amiloride, was included in some reaction mixtures at a final concentration of 0.5 mmol/L [16]. The caseinolytic areas were observed and microphotographed under dark-field illumination.

RESULTS

Design and characterization of uPA-sensitive cryptic CendR peptide

Our aim was to identify a peptide, which could be cleaved by uPA and transform into a CendR peptide. The preferred recognition and cleavage sequence of uPA is SGRSA [7]. The cleavage occurs after the arginine residue at the P1 position, making uPA capable of unmasking cryptic CendR motifs. First, we generated T7 phages that displayed peptides mimicking uPA post-cleavage sequences, and examined the binding of the resulting phages to immobilized NRP-1 b1b2 domain recombinant protein. The peptides had a C-terminal arginine residue without a CendR element, or in the context of single or tandem CendR element (SGR, RSGR, or RPARSGR) (Fig. 1A). All three peptides bound to wild-type NRP-1; the tandem CendR peptide, RPARSGR, showed the strongest binding. Only background binding was seen to NRP-1 b1b2 with a mutated CendR binding pocket (S346A-E348A-T349A) [2]. Binding of RPARSGR- phage to NRP-1 was inhibited by q-dots functionalized with the prototypic CendR peptide RPARPAR with unblocked C-terminus, but not by control q-dots with an RPARPAR peptide with a blocked C-terminus (RPARPAR-NH₂) or heptaglycine, indicating that RPARSGR shares a binding site with RPARPAR (Fig. S1). Phage displaying non-processed, cryptic CendR peptides (SGRSAGGSVA, RSGRSAGGSVA, or RPARSGRSAGGSVA) did not bind to NRP-1 (Fig. S2). When exposed to recombinant uPA, RPARSGRSAGGSVA phage binding to recombinant NRP-1 b1b2 was increased to the level seen with RPARSGR phage, indicating CendR activation (Fig. 1B). Also, treatment of uCendR phage with trypsin, which cleaves peptides after basic residues and can activate cryptic CendR elements [2], resulted in elevated NRP-1 binding, whereas control proteases thrombin and collagenase-I had no effect (Fig. 1B). We chose RPARSGR↓SAGGSVA (designating it uCendR) and its product RPARSGR (uCendR-X) for further studies.

Mass spectroscopy demonstrated that uCendR peptide was cleaved by uPA at the position after the RPARSGR (Figs. 1C and 2C), as was anticipated based on uPA specificity [7]. Cleavage of uCendR peptide on phage by uPA was dose- and time-dependent (Fig. 1D, Fig. S3) and was inhibited by the uPA inhibitor, uPA-Stop (Fig. S4). These results show that processing of uCendR peptide by uPA converts the peptide from inert to active peptide capable of binding to the CendR binding pocket of NRP-1 and to in NRP-1-positive cells.

Tumor targeting of nanoparticles with uCendR peptide

Short homing peptides identified using *in vivo* phage biopanning typically have K_d's in the low micromolar range and are best-suited for targeted delivery of nanoparticles because avidity effects of multivalent presentation on nanoparticles makes up for the modest affinity.

To determine if uCendR could be used for tumor delivery of synthetic nanosized carriers, we tested the peptides coated onto AgNPs (Fig. 2) [13]. We prepared ~50 nm AgNPs, approximating the size of the T7 phage, and coated the silver core with PEGylated-neutravidin (NA), as previously described [13]. Biotinylated uCendR and control peptides were then loaded on the NA-AgNPs (200 peptides per particle, which is comparable to peptide density on the phage coat) and the constructs were applied to cultured PPC-1 cells, known to overexpress NRP-1, or to M21 control cells that are negative for NRP-1 expression (Fig. 2). Dark-field (light-scattering) imaging showed that uCendR-AgNPs did not bind PPC-1 cells, but if pre-incubated with uPA, acquired strong affinity towards the cells (Fig. 2B). NRP-1-negative M21 cells did not bind the uPA-treated uCendR-AgNPs, supporting a conclusion that the cleaved peptide acquired receptor specificity. By utilizing Ag etching chemistry to dissolve extracellular (free and cell surface-bound) nanoparticles [13] we observed that a fraction of the protease-cleaved uCendR AgNPs had internalized into the cells (Fig. 2C), suggesting NRP-1-dependent endocytosis [17].

To confirm that uPA cleaves uCendR on the AgNPs, we tested biotin-Ahx-GGSGRPARSGR↓SAGGK(Rho)DA, in which a rhodamine B dye (Rho) and biotin are positioned at opposite ends of the peptide. We loaded this peptide onto AgNPs carrying NA labeled with Oregon Green 488 (OR488), which should not be removed from the NPs by uPA (Fig. 2F).

Both Rho and OR488 were partially quenched when loaded onto NA-AgNPs. This effect allowed us to monitor protease cleavage, which was expected to free the dye and restore its fluorescence intensity. Incubation of Rho-uCendR-AgNPs-OR488 with uPA caused an increase in fluorescence in both the Rho and OR488 channels (Fig. 2G). After centrifuging and pelleting the particles, the supernatant showed significant Rho fluorescence. In a control experiment, we prepared NA-AgNPs with free biotin added prior to the biotinylated Rho peptide in order to block loading. In this sample the exposure to uPA did not cause any change in OR488 fluorescence, consistent with the NA protein being stable to uPA proteolysis. These results show that uPA proteolytic processing of the synthetic uCendR peptide can occur on AgNPs, and that it results in an active CendR peptide being displayed that is capable of cellular binding and internalization.

uCendR peptide homes to breast tumors

During solid tumor growth, tissue remodeling, and angiogenesis uPA activity is upregulated in both stromal and malignant cells. In agreement with reported high expression of uPA by 4T1 tumors [18], *in situ* zymography on unfixed cryosections of 4T1 tumors demonstrated the presence of a caseinolytic plasminogen activator activity that was greatly reduced by inclusion of uPA inhibitor amiloride in the overlay mix (Fig. S5). We synthesized a fluorescein-labeled uCendR (FAM-uCendR) and injected the peptide intravenously into mice bearing syngeneic orthotopic 4T1 breast tumors. FAM-uCendR accumulated in tumor, but not normal tissues, with tumor tissue being fluorescent under blue light (Fig. 3a). No signal was seen in mice injected with FAM-conjugated control peptide. Confocal microscopy revealed accumulation of FAM-uCendR peptide in and around tumor vessels and in tumor parenchyma (Fig. 3C,D). To explore the potential of uCendR peptide for *in*

vivo targeting of nanoparticles, we injected 4T1 tumor mice intravenously with the AgNPs coated with uCendR, uCendR-X, or the control peptide. The circulation half-life of the nanoparticles is 2 hrs, determined by analyzing Ag content in blood (Nu Instruments AtomM) [13]. After 4 h circulation, the mice were perfused with biocompatible etchant that removes extracellular AgNP, and tissue sections were examined by autometallographic silver staining (Fig. 4) [13]. Both the uCendR-AgNPs and uCendR-X-AgNPs were found in the tumor, as well as in the liver, an organ of the reticuloendothelial system involved in nanoparticle clearance. Internalization of control AgNPs into tumor cells was relatively low (Fig. 4C). Notably, uCendR-X AgNPs accumulated in the lung and heart (Fig. 4B,F,H), sites where the uCendR AgNPs showed minimal accumulation, suggesting uCendR has an improved tumor selectivity (Fig. 4A,E,G). Active CendR peptides are known to bind to the vascular beds in the lung and in the heart, due to a combination of NRP-1 expression in those tissues, and heart and lung vessels being the first capillary vascular beds encountered by intravenously-administered compounds [2,19]. Our observation is consistent with low uPA expression in resting tissues, including heart and lung. Our data suggest that uCendR coating can improve tumor uptake of nanocarriers, while reducing off-target accumulation relative to active CendR coating.

uCendR peptide is potentiated by cell surface recruitment

iRGD must be recruited to angiogenic integrins for efficient cell surface proteolytic processing to expose the cell- and tissue penetrating RGDK CendR fragment [1]. We reasoned that recruitment to the cell surface – a proteolytically privileged site – may also enhance the activation of uCendR peptide. In the cell surface microenvironment, angiogenic integrins are known to interact both with high-affinity uPA receptor uPAR and with NRP-1 [20, 21]. To achieve cellular recruitment of the peptide we examined uCendR peptide coupled to α_v integrin-binding CRGDC motif (uCendR-RGD configuration: RPARSGR↓SAGGSVACRGDC). We first studied whether the CendR and RGD modules in the composite uCendR-RGD peptide retain their biological activities on cultured cells. Addition of the CRGDC module of uCendR-RGD caused phage binding to M21 melanoma cells positive for α_v integrins, but not to M21L cells, a sub-line of M21 that lacks α_v integrin expression [22] (Fig. S6A). M21 melanoma cells, negative for cell surface NRP-1 [2], remained negative for uCendR-X phage binding (Fig. S6A). Also, no M21 binding was seen for the derivative of uCendR-RGD phage with mutated CRGDC motif (CRGEC, known to dramatically reduce integrin binding). We then confirmed that the uCendR module in uCendR-RGD phage was C-terminally exposed after exogenous uPA addition, and that processing was enzyme concentration and time dependent (Fig. S6B). Finally, cultured PPC1 cells that were inefficient in taking up uCendR phage bound and internalized uCendR-RGD phage in a time-dependent manner (Fig. S6C–E). Thus, the cellular binding and entry of the uCendR peptide can be enhanced by cell surface recruitment following a multistep activation pathway similar to the iRGD peptide [1].

The ability of iRGD and other TPPs to penetrate freshly excised live tumor samples correlates with their systemic tumor penetration activity *in vivo* [23]. The *ex vivo* tumor penetration assay (tumor “dipping assay”) has been applied for studying the mechanism of tumor penetration [23], and is potentially useful for evaluation of TPP for their translational

potential since the assay can be carried out on clinical tumor samples [33]. Excised mouse tumor xenografts were dipped in a solution of uCendR and uCendR-RGD phages to investigate penetration (Fig. 5A–D). The phage particles were found within the tumor rim and deeper regions of the tumor tissue. Next, we tested freshly excised appendiceal cancer samples with the dipping assay and assessed the uCendR-RGD phage and control phages (iRGD, RPARPAR and G7) (Fig. 5E–G). We observed penetration of the malignant tissue for uCendR-RGD and positive controls (iRGD and RPARPAR) whereas no heptaglycine (G7) phage was detectable inside the clinical tumor samples underlying potential translational potential of the uCendR-RGD peptide. These studies demonstrate that the uCendR activation by endogenous uPA can be enhanced by cell surface recruitment and underline the translational potential of the uCendR-RGD peptides for targeted payload delivery.

DISCUSSION

Here we describe a strategy for developing novel tumor penetrating peptides that are activated by tumor-derived proteases. Our approach is based on engineering a cryptic CendR motif in the recognition and cleavage site of a tumor protease, such that proteolytic processing results in C-terminal exposure of a NRP-1-interacting CendR motif. For proof-of-concept studies we use urokinase – a tumor associated serine endoprotease widely accepted to be a key mediator of tumor cell invasion and tumor tissue remodeling. We show that a uPA-sensitive CendR peptide (uCendR) is effectively and specifically processed to active CendR peptide that is able to bind to recombinant and cell surface NRP-1 and that activated uCendR peptide is capable of targeting nanoparticle payloads to immobilized neuropilin and cells overexpressing NRP-1. When injected systemically in mice bearing orthotopic breast tumor xenografts, uCendR peptide is processed to active CendR peptide that triggers tumor-selective tissue penetration, resulting in about 5 times more tumor accumulation of uCendR nanoparticles compared to nanoparticles functionalized with a control peptide. Finally, we show that cell surface recruitment by angiogenic integrin-binding CRGDC module can further potentiate uCendR activity, probably through a combination of concentrating the uCendR peptide at proteolytically privileged cell surface microenvironment, and by concentrating the uCendR in the proximity of NRP-1 that allows efficient activation of cell and tissue penetration pathway. The uCendR platform can be used for delivery of conjugated low molecular weight cargo (fluorophores) and nanoparticles (phages and AgNP). Our studies with the prototypic tumor penetrating peptide, iRGD, demonstrate the suitability of CendR peptides for delivery of conjugated anticancer and imaging agents of different classes, including low molecular weight drugs, antibodies, and nanoparticles. A remarkable property of iRGD is to trigger a transient tumor specific bulk penetration pathway that sweeps co-injected compounds into extravascular tumor tissue. It remains to be addressed in follow-up studies if and to what extent the uCendR and uCendR-RGD peptides are capable of triggering the tumor-specific bystander effect. The mechanism of the tissue penetration of the nanoparticles functionalized with CendR peptides remains a subject of intense research efforts. CendR is an active transport process that requires energy [1]. It is not limited to extravasation, but also includes penetration of tumor parenchyma. Cell-to-cell transport of CendR payloads has been demonstrated [17], which may be the

basis of the trans-tissue transport. However, we cannot exclude a contribution from neuropilin-mediated increase in vascular and tissue permeability, although the speed of the transport does not agree with a passive diffusion process [23].

Other laboratories have reported development of protease-actuated conditional targeting systems. The Tsien lab has developed activatable cell penetrating peptides (CPP), in which polycationic cell penetration domain is linked via a protease-cleavable linker to a polyanionic inhibitory domain [24–26]. In the presence of tumor-derived proteases, such as MMP-2 or -9, the inhibitory domain is released and cell penetrating domain is exposed to internalize in adjacent cells. Interestingly, recent work from our laboratory demonstrated the convergence of a heparan sulfate (HS)-dependent pathway of CPP internalization with the CendR mechanism [27]. Thus activatable CPP with uCendR or another linker designed to be cleaved by a tumor protease, after a basic residue, may exploit both HS and CendR pathways. An alternative approach to achieve conditional protease-activated targeting is to coat nanoparticles with a protease-sensitive PEG shield, e.g. using PEG-MMP-2 cleavable peptide-lipid liposomes for hepatocellular carcinoma-selective targeting [28].

CendR peptides interact with NRP-1 and -2 and this binding is not species specific. CendR-neuropilin interaction triggers cellular internalization in cells of both human and mouse origin, and *ex vivo* penetration of experimental mouse tumors and human tumors. Neuropilins are overexpressed in most solid tumors, in both malignant cells and in cells of tumor stroma (e.g. endothelial cells, macrophages, fibroblasts). The human degradome comprises about 600 proteases, many of which are upregulated in tumors and have substrate specificities compatible with unmasking cryptic CendR motifs. For example, hepsin [29,30] and matriptase [31–33] cleave after basic residues and their activity is upregulated in tumors [30,32]. A design strategy, similar to what we have developed here for uCendR peptide, could be readily adopted to construct translationally relevant tumor penetrating peptides activated by these proteases.

CONCLUSIONS

We have developed a designer tumor penetrating peptide that is activated by a tumor protease, urokinase. This approach can be used to expand the arsenal of tumor penetrating CendR peptides by rational design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Sugahara KN, et al. Tissue-penetrating delivery of compounds and nanoparticles into tumors. *Cancer Cell*. 2009; 16:510–20. [PubMed: 19962669]
2. Teesalu T, et al. C-end rule peptides mediate neuropilin-1-dependent cell, vascular, and tissue penetration. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:16157–62. [PubMed: 19805273]
3. Sugahara KN, et al. Tumor-penetrating iRGD peptide inhibits metastasis. *Molecular cancer therapeutics*. 2015; 14:120–8. [PubMed: 25392370]
4. Atkinson JM, Siller CS, Gill JH. Tumour endoproteases: the cutting edge of cancer drug delivery? *Br J Pharmacol*. 2008; 153:1344–52. [PubMed: 18204490]
5. Edwards, DR., et al., editors. *The cancer degradome: proteases and cancer biology/Dylan*. Vol. xxiii. New York, NY: Springer; 2008. p. 926
6. Andreasen PA, Egelund R, Petersen HH. The plasminogen activation system in tumor growth, invasion, and metastasis. *Cell Mol Life Sci*. 2000; 57:25–40. [PubMed: 10949579]
7. Ke SH, et al. Optimal subsite occupancy and design of a selective inhibitor of urokinase. *J Biol Chem*. 1997; 272:20456–62. [PubMed: 9252355]
8. Blasi F. uPA, uPAR, PAI-1: key intersection of proteolytic, adhesive and chemotactic highways? *Immunology today*. 1997; 18:415–7. [PubMed: 9293155]
9. Liu S, Bugge TH, Leppla SH. Targeting of tumor cells by cell surface urokinase plasminogen activator-dependent anthrax toxin. *J Biol Chem*. 2001; 276:17976–84. [PubMed: 11278833]
10. Rono B, et al. Antitumor efficacy of a urokinase activation-dependent anthrax toxin. *Mol Cancer Ther*. 2006; 5:89–96. [PubMed: 16432166]
11. Gerspach J, et al. Target-selective activation of a TNF prodrug by urokinase-type plasminogen activator (uPA) mediated proteolytic processing at the cell surface. *Cancer Immunol Immunother*. 2006; 55:1590–600. [PubMed: 16636812]
12. Chung DE, Kratz F. Development of a novel albumin-binding prodrug that is cleaved by urokinase-type-plasminogen activator (uPA). *Bioorg Med Chem Lett*. 2006; 16:5157–63. [PubMed: 16875815]
13. Braun GB, et al. Etchable plasmonic nanoparticle probes to image and quantify cellular internalization. *Nature materials*. 2014; 13:904–11. [PubMed: 24907927]
14. Teesalu T, Sugahara KN, Ruoslahti E. Mapping of vascular ZIP codes by phage display. *Methods in enzymology*. 2012; 503:35–56. [PubMed: 22230564]
15. Sappino AP, et al. Differential protease expression by cutaneous squamous and basal cell carcinomas. *The Journal of clinical investigation*. 1991; 88:1073–9. [PubMed: 1918364]
16. Teesalu T, Blasi F, Talarico D. Embryo implantation in mouse: fetomaternal coordination in the pattern of expression of uPA, uPAR, PAI-1 and alpha 2MR/LRP genes. *Mech Dev*. 1996; 56:103–16. [PubMed: 8798151]
17. Pang HB, et al. An endocytosis pathway initiated through neuropilin-1 and regulated by nutrient availability. *Nature communications*. 2014; 5:4904.
18. Mitra SK, et al. Intrinsic focal adhesion kinase activity controls orthotopic breast carcinoma metastasis via the regulation of urokinase plasminogen activator expression in a syngeneic tumor model. *Oncogene*. 2006; 25:4429–40. [PubMed: 16547501]
19. Zhang L, Hoffman JA, Ruoslahti E. Molecular profiling of heart endothelial cells. *Circulation*. 2005; 112:1601–11. [PubMed: 16144998]
20. Degryse B, et al. Domain 2 of the urokinase receptor contains an integrin-interacting epitope with intrinsic signaling activity: generation of a new integrin inhibitor. *The Journal of biological chemistry*. 2005; 280:24792–803. [PubMed: 15863511]
21. Bagri A, Tessier-Lavigne M, Watts RJ. Neuropilins in tumor biology. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2009; 15:1860–4. [PubMed: 19240167]
22. Petitclerc E, et al. Integrin alpha(v)beta3 promotes M21 melanoma growth in human skin by regulating tumor cell survival. *Cancer research*. 1999; 59:2724–30. [PubMed: 10363998]

23. Sugahara KN, et al. Coadministration of a tumor-penetrating peptide enhances the efficacy of cancer drugs. *Science*. 2010; 328:1031–5. [PubMed: 20378772]
24. Jiang T, et al. Tumor imaging by means of proteolytic activation of cell-penetrating peptides. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:17867–72. [PubMed: 15601762]
25. Aguilera TA, et al. Systemic *in vivo* distribution of activatable cell penetrating peptides is superior to that of cell penetrating peptides. *Integrative biology: quantitative biosciences from nano to macro*. 2009; 1:371–81. [PubMed: 20023744]
26. Nguyen QT, et al. Surgery with molecular fluorescence imaging using activatable cell-penetrating peptides decreases residual cancer and improves survival. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:4317–22. [PubMed: 20160097]
27. Pang HB, Braun GB, Ruoslahti E. Neuropilin-1 and heparan sulfate proteoglycans cooperate in cellular uptake of nanoparticles functionalized with cationic cell-penetrating peptides. *Sci Adv*. 2015; 1:e1500821. [PubMed: 26601141]
28. Zhu L, Kate P, Torchilin VP. Matrix Metalloprotease-2-responsive multifunctional liposomal nanocarrier for enhanced tumor targeting. *ACS Nano*. 2012; 6:3491–3498. [PubMed: 22409425]
29. Herter S, et al. Hepatocyte growth factor is a preferred *in vitro* substrate for human hepsin, a membrane-anchored serine protease implicated in prostate and ovarian cancers. *Biochem J*. 2005; 390:125–36. [PubMed: 15839837]
30. Wu Q, Parry G. Hepsin and prostate cancer. *Front Biosci*. 2007; 12:5052–9. [PubMed: 17569629]
31. Lee SL, Dickson RB, Lin CY. Activation of hepatocyte growth factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease. *J Biol Chem*. 2000; 275:36720–5. [PubMed: 10962009]
32. List K, Bugge TH, Szabo R. Matriptase: potent proteolysis on the cell surface. *Mol Med*. 2006; 12:1–7. [PubMed: 16838070]
33. Uhland K. Matriptase and its putative role in cancer. *Cell Mol Life Sci*. 2006; 63:2968–78. [PubMed: 17131055]
33. Sugahara KN, et al. A tumor-penetrating peptide enhances circulation-independent targeting of peritoneal carcinomatosis. *J Control Release*. 2015; 212:59–69. [PubMed: 26071630]

Figure 1. Proteolytic activation of uCendR peptide

(A) Binding of T7 phages that display peptides mimicking uPA post-cleavage sequences to wild-type (wt) and mutant NRP-1 b1b2 domain. Prototypic CendR peptide RPARPAR served as a positive control. Note that binding is increased with tandem CendR elements. Phage binding is expressed as fold control phage displaying heptaglycine. (B) Effect of protease pretreatment uCendR phage binding. uCendR-X (black column) serves as a positive control. (C) Mass spectroscopy analysis of uCendR peptide processing. Note that uPA treatment results in near-complete quantitative conversion of the uCendR peptide to uCendR-X peptide. (D) uPA pretreatment (red graph) dose-dependently induces uCendR phage binding to the NRP-1 overexpressing PPC1 cells at 4°C. uCendR-X (black column) serves as a positive control for binding. N = 3, mean±s.d., *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 2. Targeting uCendR-nanoparticles with uPA activation

(A) AgNPs were coated with neutravidin, and loaded with the uCendR peptide to form uCendR-AgNPs. PPC-1 binding was elicited by proteolytic activation of AgNPs with uPA, and visualized by live cell dark field microscopy. (B) uPA addition stimulates PPC-1 binding. (C) Extracellular Ag etching indicates that a fraction of AgNPs was internalized into the cells. (D) Without uPA, no binding was observed. (E) NRP-1-negative M21 cells do not bind uPA-activated uCendR-AgNPs. (F) Schematic of the FRET assay employing rhodamine (Rho)-labeled biotin peptide loaded into Oregon Green 488 (OR488)-labeled neutravidin (NA) on AgNPs. In this initial state, both OR488 and Rho are quenched. Addition of uPA enzyme causes cleavage of the Rho-containing peptide fragment, causing unquenching of the two dyes. (G) Fluorescence increases in both Rho and OR488 channels, when the peptide conjugates are incubated with uPA. Free biotin blocked the loading of the peptide, and those samples showed no change in OR488 signal when exposed to uPA. N=3, mean $\pm$ s.d.

Figure 3. *In vivo* tumor homing of FAM- labeled uCendR peptide
 (A,B,E,F) 200 μ g of FAM-uCendR or control FAM-ARAL peptide (ARALPSQRSR-NH₂) in PBS was intravenously injected into mice bearing syngeneic orthotopic 4T1 breast tumors. The peptides were allowed to circulate for 2 h, animals were perfused to wash out unbound peptide, and the organs were collected and viewed under blue light. Arrows point to the FAM-peptide accumulation in tumors. (C,D,G,H) Confocal images of cryosections of orthotopic 4T1 tumor mice injected with uCendR (C,D) or control ARAL (G,H) peptides and processed for immunostaining. Immunofluorescence of CD31 (vascular endothelial cells) is in red and nuclei were stained with DAPI (blue). Arrows point to uCendR peptide-positive cells in tumor parenchyma. b, brain; lu, lung; h, heart; li, liver; s, spleen; p, pancreas; k, kidney; t, tumor. Representative fields from multiple sections of tumors (N = 3) are shown. Scale bars = 50 μ m.

Figure 4. In-vivo AgNP biodistribution

Dark field histological autometallography imaging of tissue sections from AgNP-injected mice shows that tumor homing specificity is improved for uCendR relative to uCendR-X. Silver signal appears as white on a black background. AgNPs were functionalized with either uCendR, uCendR-X, or control peptide, each injected intravenously into 4T1 breast tumor-bearing mice. The “no Ag” control set of mice received no injection. Perfusion with extracellular Ag-etching solution was performed after 4 h circulation. Representative images of tumor (A–D), lung (E,F), heart (G,H), and liver (I,J), shows that uCendR-AgNPs accumulate primarily in tumor and liver. uCendR-X-AgNPs shows signal in tumor, lung, heart, and liver. Control peptide AgNPs showed minimal tumor signal above the “no Ag” control. K–L) Quantification of signal as percent of pixels with intensity above threshold, indicates uCendR and uCendR-X have similar tumor homing capability. Organ specificity was greater with uCendR. (N=4, except N=3 for “no Ag”; mean±s.d., *p < 0.05; **p < 0.01; ***p < 0.001, n.s. = not significant). Scale bars are 50 μm.

Figure 5. uCendR phage penetration into tumor tissue explants in *ex vivo* tumor penetration assay

(A–D) Orthotopic 4T1 breast cancer xenografts in syngeneic Balb/c mice were excised and maintained in short-term culture containing 10^9 pfu/ml of phage expressing indicated peptides followed by tumor washing, fixation and immunofluorescence staining with an anti-T7 phage antibody (green), an anti-CD31 antibody (red), and DAPI, and confocal imaging. The dotted lines indicate the edge of the tumor. uCendR (A), RPARPAR (B), and uCendR-IRGD (C) peptide phage shows penetration into the tumor explants both at the tumor periphery (arrowheads in C) and in deep tumor parenchyma (arrows in C). (E–G) Tumor dipping assay on human appendiceal cancer explants was carried out as above, except tissue sections were not stained with anti CD31 antibody. Note that whereas both uPA-dependent CendR peptide and active prototypic CendR peptides RPARPAR penetrate into the malignant tissue, the control is negative. Scale bars: A–D: 350 μ m; E–G: 100 μ m.