Mechanistic Analysis of Cellular Internalization of a Cell- and Skin-Penetrating Peptide

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Abstract

Background Keratinocytes are a targeted cell-type for treating skin disease. However, delivering drugs into the skin and keratinocytes remains challenging due to transport barriers posed by both the stratum corneum and cell membrane. Nonetheless, SPACETM peptide has been shown effective in delivering a number of macromolecules into the skin and cells. Accordingly, we sought to explore the mechanism of SPACETM peptide internalization under conditions relevant for skin delivery.

Methods Mechanistic analysis was performed using live cell confocal microscopy. Potential binding targets were assessed by Western blot and mass spectrometry.

Results Experimental data suggest an energy-dependent mechanism for the cellular entry of SPACETM peptide into human epidermal keratinocytes. SPACETM peptide associates with the cell membrane within seconds and then is internalized rapidly. Co-incubation with heparin sulfate or serum suggests that association with the cell membrane may be specific in nature. Western blot and mass spectrometry studies are consistent with earlier findings that keratin may play an important role in SPACETM peptide internalization.

Conclusions This work highlights the potential for delivering drug into the cytoplasm of keratinocytes for the treatment of skin disease through topical application. Further, it highlights an ongoing need for studies involving cell-penetrating peptides under clinically relevant conditions.

Lay Summary SPACETM peptide has been shown to enhance delivery of a wide variety of macromolecules into the skin and cells. However, the mechanism of cell entry of SPACETM peptide under conditions relevant for skin delivery is not understood. Accordingly, we sought to explore the mechanism of SPACETM peptide internalization in keratinocytes under conditions relevant for skin delivery. A better understanding of the dominating pathways and major molecular players involved in SPACETM peptide internalization under clinically relevant conditions will aid the development and translation of SPACETM peptide-based technologies for treating skin disease.

Keywords SPACETM peptide · Cell-penetrating peptide · Skin · Cell internalization · Drug delivery

Abbreviations

SC	Stratum corneum
FITC	Fluorescein isothiocyanate
HEKa	Human adult epidermal keratinocyte
HBSS	Hank's balanced salt solution
EtBr	Ethidium bromide
FBS	Fetal bovine serum
TAMRA	Tetramethylrhodamine
EIPA	5-(N-Ethyl-N-isopropyl)amiloride
M β CD	Methyl- β -cyclodextrin
EGF	Epidermal growth factor
CPP	Cell-penetrating peptide

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Introduction

Keratinocytes are the major cell-type present in human viable skin tissue [1], and therefore, are considered a primary target for treating skin-resident disease [1, 2]. Several

dermatological conditions including psoriasis [2], atopic dermatitis [3], wound healing [4, 5], and bullous impetigo [6] manifest clinical symptoms due to abnormal keratinocyte function or behavior, and therapies that target keratinocytes have shown promise [7, 8]. Unfortunately, however, delivery of drugs into keratinocytes is inherently difficult. Systemic approaches for drug delivery to keratinocytes are limited by drug degradation in the GI tract, limited accumulation in the dermis and epidermis, and systemic toxicity [9]. Topical delivery methods can potentially address this challenge; however, cutaneous drug delivery remains difficult due to barrier properties of the outermost layer of the skin—the stratum corneum (SC) [10]. Further, the cell membrane of keratinocytes can pose an additional significant transport barrier, and intracellular sequestration by trafficking machinery can severely limit bioavailability at the drug target site [11].

To overcome these challenges, peptides have emerged as a promising strategy for simultaneous enhancement of drugs across multiple biological barriers including the SC and cell membrane [12–15]. In particular, SPACE™ peptide has been demonstrated effective at delivering a wide variety of macromolecules including cyclosporine A [16], hyaluronic acid [17], and siRNA [18] into skin and cells, and extensive efforts have been spent on identifying its mechanism of skin penetration enhancement [19]. However, the mechanism of cell entry of SPACE™ peptide under conditions relevant for skin delivery is not understood. Accordingly, we sought to explore the mechanism of SPACE™ peptide internalization in keratinocytes under conditions relevant for skin delivery. A better understanding of the dominating pathways and major molecular players involved in SPACE™ peptide internalization under clinically relevant conditions will aid the development and translation of SPACE™ peptide-based technologies for treating skin disease. Here, we report evidence of an energy-dependent mechanism for drug delivery by SPACE™ peptide directly into the cytoplasm of human epidermal keratinocytes.

Materials and Methods

SPACE™ Peptide

Fluorescein isothiocyanate (FITC) was used as a model membrane-impermeable small molecule drug. FITC-labeled SPACE™ peptide (FITC-AC-TGSTQHQ-CG, Disulfide Bridge 2–10) was purchased from RS Synthesis, LLC (Louisville, KY). Final composition and purity was confirmed by RS Synthesis, LLC prior to shipment. FITC-SPACE™ peptide composition was determined by mass spectrometry, and peptide purity of 99.1 % was determined by HPLC.

Cell Culture

Human adult epidermal keratinocytes (HEKa cells) and cell culture materials were acquired from Life Technologies (Grand Island, NY). HEKa cells were cultured in 1× keratinocyte serum-free medium supplemented with 25 U/mL penicillin, 25 µg/mL streptomycin, and 50 µg/mL neomycin. Cultures were grown at 37 °C with 5 % CO₂.

Cell Internalization

Cells were seeded on poly-d-lysine-coated glass bottom culture dishes (MatTek Corporation, Ashland, MA) and were allowed to attach and proliferate until ~80 % confluency under standard culture conditions (37 °C with 5 % CO₂). Then, media was removed and 50 µL of fluorescent peptide solution in culture media was added to the cell culture dish. As a control, an equivalent amount of media was added in place of peptide solution. Cells were incubated at 37 °C unless otherwise specified, after which the fluorescent solution was removed and cells were prepared for confocal microscopy.

Confocal Microscopy

After incubation with FITC-SPACE™ peptide or control solution, cells were incubated with 5 µg/mL Hoechst 33342 (Life Technologies, Grand Island, NY) for 5 min at room temperature and then washed with supplemented Hank's Balanced Salt Solution (HBSS, Lonza Group Ltd., Basel, Switzerland). HBSS was supplemented with 200 µM Trolox (Sigma, St. Louis, MO) to limit phototoxicity and photobleaching during imaging. Cells were washed with supplemented HBSS three times for 5 min each. The cell culture dishes were then filled with 90 µM ethidium bromide (EtBr, Life Technologies, Grand Island, NY) in supplemented HBSS and imaged using a confocal laser scanning microscope (Olympus Fluoview 1000S, Olympus, Center Valley, PA); 90 µM EtBr was sufficient to quench any significant fluorescence from externally bound FITC-SPACE™ peptide. All instrument settings were kept constant to enable comparisons between experimental conditions, and a ×30 silicon immersion objective was used to capture the entire thickness of the cell. Dead cells indicated by EtBr staining of the nucleus were ignored during analysis.

Image Analysis

Image analysis was performed using Fiji to determine the overall fluorescence intensity for each cell. For localization studies a ×60 silicon immersion objective was used. Five to seven slices were taken at 500-µm intervals and analyzed in CellProfiler [20]. Briefly, unaltered greyscale confocal images were loaded. Optimized-thresholding was used to segment

cell nuclei. The cell outline was then determined by expanding from the centroids of the cell nuclei until the variance between the fluorescence intensities of the cell and local background was minimized. Lastly, vesicles were segmented using an optimized-thresholding algorithm with size, shape, and position constraints. After segmentation, controls were subtracted and various quantitative information, e.g., vesicle intensities, cytoplasm intensity, vesicle location, and vesicle distribution from nucleus were collected and analyzed.

Membrane Association

SPACE™ peptide association with the cell membrane was assessed with confocal microscopy. Nuclei were stained with Hoechst 33342, and cells were focused on using brightfield and Hoechst channel. The PMT for the FITC channel was adjusted, so 10 mg/mL FITC-SPACE™ peptide in solution was not detected. Then, 10 mg/mL FITC-SPACE™ peptide was added to the culture dish and images were taken every 2 s for 2 min.

The specificity of SPACE™ peptide association with the cell membrane was explored by co-incubation of FITC-SPACE™ peptide with molar excess heparin sulfate (Sigma-Aldrich, St. Louis, MO) or fetal bovine serum (FBS, Sigma Aldrich, St. Louis, MO). 2× molar excess of heparin sulfate was used to compete with non-specific electrostatic interactions. 10× excess weight of total protein of FBS was used to compete with non-specific protein interactions. Cells were incubated for 1 h with solutions of heparin sulfate or FBS in media. Media alone was used as a control. Simultaneously, FITC-SPACE™ peptide was incubated for 1 h with heparin sulfate or FBS. Media alone was used as a control. After 1 h incubation, media, heparin sulfate, and FBS solutions were removed from the cell culture dishes and replaced with the solutions of FITC-SPACE™ peptide incubated with heparin sulfate, FBS, or media alone. Cell internalization was then determined as described above.

Protein Purification

Protein from cell lysate was purified using the Membrane I, ReadyPrep™ Protein Extraction Kit, and 2-D Cleanup Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturers recommended protocol. Briefly, cells were detached from the culture flask with StemPro® Accutase® (Life Technologies, Grand Island, NY), centrifuged to isolate the cell pellet, and then re-suspended in extraction buffer supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO). StemPro® Accutase® is a gentle cell dissociation reagent, and was used to limit digestion of extracellular proteins during detachment. Cells were then lysed with a Misonix Sonicator® 3000 cycled on/off for 1 min each. The

cell suspension was maintained on ice to avoid overheating. After lysing, proteins were fractionated into water-soluble (e.g., cytosolic proteins), surfactant-soluble (e.g., hydrophobic proteins, membrane proteins), and insoluble (e.g., multi-transmembrane proteins, filamentous proteins) fractions. The amount of protein in each fraction was quantified using the RC DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

SDS-PAGE and Western Blot

Cell lysate fractions were diluted to equate protein concentration in each fraction and then dissolved in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) with 355 mM β -mercaptoethanol. Protein fractions were run on a Criterion™ TGX Stain-Free™ AnyKD™ Precast Gel (Bio-Rad Laboratories, Hercules, CA) following the manufacturers recommended protocol. In addition, a Precision Plus Protein™ WesternC™ standard was run to determine approximate molecular weights of sample protein bands. Ladder and all fractions were run in duplicate. After SDS-PAGE, gels were removed from their cassette. One set of in-gel ladder and fractions was set aside to provide samples for mass spectrometry. The second set was imaged using the Bio-Rad ChemiDoc™ XRS+ System and Image Lab™ software. The default stain-free gel workflow with minimal exposure was used for imaging SDS-PAGE gels to allow subsequent transfer of protein to a Western blot membrane.

After imaging, protein was transferred to a PVDF membrane using the Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories, Hercules, CA) according to the manufacturers recommended protocol. The membrane was blocked with 5 % dry milk in Tris-buffered saline with tween-20 (TBST) overnight at 4 °C. Then, the membrane was washed with TBST and incubated with 10 μ g/mL FITC-SPACE™ peptide in TBST with 1 % dry milk for 1 h. After incubation, the membrane was washed and imaged using the Bio-Rad ChemiDoc™ XRS+ System and Image Lab™ software. Default FITC filter set was used, and exposure was adjusted automatically for the best resolution of protein bands with bound FITC-SPACE™ peptide. After imaging, the unused SDS-PAGE samples were aligned with the PVDF membrane, and gel bands corresponding to FITC-SPACE™ peptide binding were cutout with a sterile surgical scalpel and stored in 1 mL ddH₂O in individual microcentrifuge tubes for analysis via mass spectrometry.

Mass Spectrometry

In-gel digest, mass spectrometry, and all analysis were performed by MS Bioworks LLC (Ann Arbor, MI).

Co-Incubation with Dextran

Co-incubation studies were performed with tetramethylrhodamine-labeled 3000 Da dextran (TAMRA-dextran, Life Technologies, Grand Island, NY). 5 mg/mL TAMRA-dextran was incubated with 10 mg/mL FITC-SPACE™ peptide. 5 mg/mL TAMRA-dextran alone and 10 mg/mL FITC-SPACE™ peptide alone were used as controls. Cell internalization and intracellular localization were determined as described above.

Endocytosis Inhibitors

The mechanism of SPACE™ peptide internalization was explored using endocytosis inhibitors that were previously identified in the literature [21]. Endocytosis inhibitors were acquired from Sigma-Aldrich (St. Louis, MO). Cells were incubated with various endocytosis inhibitors, or at 4 °C, for 1 h prior to the addition of fluorescently labeled peptide. 5-(N-Ethyl-N-isopropyl)amiloride (EIPA), cytochalasin D, nocodazole, dynasore, genistein, and monodansylcadaverine were dissolved in DMSO and then diluted in media to a concentration of 100 µM, 1 µg/mL, 5 µg/mL, 25 µg/mL, 200 µg/mL, and 200 µM, respectively. Chlorpromazine, nystatin, methyl-β-cyclodextrin (MβCD), primaquine, and epidermal growth factor (EGF) were dissolved in media and used at concentrations of 10 µg/mL, 25 µg/mL, 10 mg/mL, 250 µg/mL, and 100 µg/mL, respectively. After incubation in the presence of inhibitors for 1 h, a solution of inhibitor at their respective concentration and FITC-SPACE™ peptide at 10 mg/mL was added to the culture dish and cell internalization was determined as described above. These studies were also performed using FITC-transferrin (50 µg/mL, Life Technologies, Grand Island, NY) as a model marker of clathrin-mediated endocytosis.

Visualization of Actin and Tubulin

Actin and microtubules were visualized with confocal microscopy. After incubation with test or control solutions, cells were washed briefly with supplemented HBSS and then fixed for 15 min with 4 % paraformaldehyde (Boston BioProducts, Ashland, MA). After fixation, cells were again briefly washed with supplemented HBSS and then permeabilized with 0.2 % Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 5 min. For visualization of actin, cells were washed three times for 5 min each, stained with 50 nM rhodamine-phalloidin (Life Technologies, Grand Island, NY) in HBSS, washed again and stained with 5 µg/mL Hoechst 33342 in HBSS for 5 min, washed again three times for 5 min each in HBSS, mounted in ProLong® Gold (Life Technologies, Grand Island, NY), and imaged. For visualization of tubulin, cells were prepared using

tubulin-α polyclonal antibody and the Image-iT™ FX Kit with Alexa Fluor® Secondary Detection Conjugates (Life Technologies, Grand Island, NY) following the manufacturers recommended protocol. Nuclei were then stained with Hoechst 33342 and mounted in ProLong® Gold before imaging.

Statistical Analysis

Data reported are mean + SD except where otherwise noted. Statistical significance was confirmed by the two-tailed, unpaired Student's *t* test in Microsoft Excel. The level of significance was set at $p < 0.05$.

Results

Binding and Internalization of SPACE™ Peptide

Kinetics of membrane association and internalization of SPACE™ peptide by keratinocytes were assessed. These measurements were performed at peptide concentrations in the range of milligram/milliliter to reflect the concentrations of SPACE™ peptide used in previous studies that assessed therapeutic applications [16–18]. In this concentration range, SPACE™ peptide nearly instantaneously bound to the cell membrane (Fig. 1a, saturation after ~1 min), while internalization showed slower kinetics (Fig. 1b, saturation after ~30 min). Representative images are shown in Fig. 1a (ii)–(v), b (ii)–(iv) for membrane association and cell internalization, respectively. Association of SPACE™ peptide with the cell membrane appears to be specific in nature. Incubation with molar excess FBS (to inhibit non-specific protein binding) and heparin sulfate (to inhibit non-specific electrostatic interactions with the cell membrane) did not decrease internalization of SPACE™ peptide (Fig. 1c).

Potential Binding Site of SPACE™ Peptide

Potential binding target of SPACE™ peptide was identified by protein separation with SDS-PAGE (Fig. 2a) and subsequent Western blot (Fig. 2b). SPACE™ peptide is small (11-mer) and cyclic; thus, we expect its relevant binding site to be amino acid residues that are located within close proximity in the primary sequence of its binding target protein. Therefore, the binding site should not be significantly affected by denaturation. Indeed, Western blot yielded three bands corresponding to ~55, ~45, and ~15 kDa molecular weight protein products. Binding was only observed for the insoluble fraction, e.g., multi-transmembrane proteins, cytoskeletal proteins, etc. (Fig. 2b). Mass spectrometry analysis of the three fluorescent bands identified 244 potential binding partners for

Fig. 1 SPACE™ peptide association with the cell membrane saturates within 1 min, while internalization saturates after 15–30 min. Competitive binding of SPACE™ peptide with serum proteins and heparin sulfate indicates cell membrane association may be mediated by a specific binding interaction. **a** SPACE™ peptide association with the cell membrane. *i* Fluorescence intensity associated with the cell membrane. *Error bars* represent mean $\pm$ SD for $n = 3$. *ii–v* Representative images showing cell membrane association after *ii* 0 s, *iii* 30 s, *iv* 60 s, and *v* 120 s. Nuclei are shown in *blue* and FITC-SPACE™ peptide is shown in *green*. **b** SPACE™ peptide internalization. *i* Fluorescence intensity internalized. *Error bars* represent mean $\pm$ SD for $n = 3$. *ii–iv* Representative images showing internalization after *ii* 5 min, *iii* 15 min, and *iv* 60 min. **c** Internalization of FITC-SPACE™ peptide was quantified in the presence of excess fetal bovine serum (FBS) or heparin sulfate. Values were normalized to internalization of FITC-SPACE™ peptide in media only. *Error bars* represent mean $\pm$ SD for $n = 3$

SPACE™ peptide. A subset of these potential binding partners based on the counts of corresponding peptide fragments is given (Table 1).

Effect of Energy Depletion on Internalization

To assess the existence of an energy-dependent mechanism of uptake, internalization at 4 °C was compared to 37 °C (Fig. 3). Internalization was nearly completely eliminated at 4 °C (95.6 $\pm$ 2.5 % and 96.3 $\pm$ 1.0 % reduction in internalization for 15 and 60 min incubation, respectively) suggesting internalization is energy-dependent (Fig. 3a). This behavior was observed regardless of the length of incubation. Representative images showing internalization at 4 °C and 37 °C are given (Fig. 3b (i) and (ii), respectively).

Co-Localization with Dextran

Internalization of SPACE™ peptide at low concentrations (μ g/mL) was previously shown to occur through macropinocytosis [12]. Macropinocytosis has also been reported as key for internalization of other CPPs [22–26]. To explore if increased internalization and localization in the cytoplasm at higher concentrations (mg/mL) was also due to macropinocytosis, dextran was used as a marker. Dextran is considered a marker of macropinocytosis [23], and therefore, if SPACE™ peptide is internalized primarily via macropinocytosis, we would expect significant co-localization of SPACE™ peptide with dextran in macropinosomes. Similarly, if SPACE™ peptide perturbs the integrity of macropinosomes leading to endosomal escape

Fig. 2 SDS-PAGE and Western blot show SPACE™ peptide binds to insoluble protein products with ~55, ~45, and ~15 kDa molecular weights. **a** SDS-PAGE and **b** Western blot with cell lysate. Binding of FITC-SPACE™ peptide to protein lanes is indicated by arrows. Protein ladder (*lane 1*), insoluble proteins (*lane 2*), water soluble proteins (*lane 3*), and surfactant soluble proteins (*lane 4*)

into the cytosol, we would expect dextran might also escape leading to its co-localization with SPACE™ peptide in the cytosol.

Results show FITC-SPACE™ peptide indeed co-localizes with TAMRA-dextran; however, dextran remains in punctate structures while SPACE™ peptide showed mostly diffuse cytoplasmic staining (Fig. 4a, co-localization indicated by yellow arrows). Additionally, dextran internalization is significantly decreased (38.1 ± 12.6 %) when co-incubated with SPACE™ peptide (Fig. 4b (i), closed bar). Internalization of SPACE™ peptide, however, was not significantly affected by co-incubation with dextran (Fig. 4b (ii), closed bar). Together, the data suggest that at least a part of SPACE™ peptide is internalized by macropinocytosis and competes with dextran internalization via the same pathway.

The occurrence of macropinocytosis was further assessed by observing membrane ruffling (Fig. 5a). Membrane ruffling is characteristic of macropinocytosis stimulation [23]. Surprisingly, SPACE™ peptide did not initiate actin rearrangement characteristic of macropinocytosis stimulation (Fig. 5a (ii), ruffling indicated by yellow arrows). Moreover, SPACE™ peptide internalization actually increased significantly (2.4 ± 0.5 fold) when macropinocytosis was inhibited with EIPA (Fig. 5b (ii), open bar). Activation of macropinocytosis with EGF did result in membrane ruffling (Fig. 5a (iv), membrane ruffling indicated by yellow arrows) but had no significant effect on SPACE™ peptide internalization (Fig. 5b (ii), closed bar). Dextran was used as a control. As expected, dextran did not stimulate micropinocytosis; however,

Table 1 Subset of potential proteins identified by mass spectrometry

Identified proteins	Accession number	Molecular weight (kDa)
Plectin	sp Q15149 PLEC_HUMAN	532
Keratin, type II cytoskeletal 5	sp P13647 K2C5_HUMAN (+1)	62
Collagen alpha-1 (XVII) chain	sp Q9UMD9 COHA1_HUMAN	150
Vimentin	sp P08670 VIME_HUMAN	54
Keratin, type II cytoskeletal 1	sp P04264 K2C1_HUMAN	66
Keratin, type II cytoskeletal 6A	sp P02538 K2C6A_HUMAN (+1)	60
Keratin, type I cytoskeletal 14	sp P02533 K1C14_HUMAN (+1)	52
Prelamin-A/C	sp P02545 LMNA_HUMAN	74
Tubulin alpha-4A chain	sp P68366 TBA4A_HUMAN	50
Actin, cytoplasmic 1	sp P60709 ACTB_HUMAN	42
Keratin, type I cytoskeletal 16	sp P08779 K1C16_HUMAN (+2)	51
Tubulin alpha-1B chain	sp P68363 TBA1B_HUMAN	50
Keratin, type II cytoskeletal 6B	sp P04259 K2C6B_HUMAN	60

Fig. 3 SPACE™ peptide internalization remains energy-dependent at high concentrations. **a** SPACE™ peptide internalization was quantified at 4 and 37 °C. A significant reduction in the amount internalized was observed at 4 °C compared to 37 °C for both 15 min incubation (open bars) and 60-minute incubation (closed bars). Error bars represent mean + SD for $n=3$. $*p<0.01$. **b** Representative images of FITC-SPACE™ peptide internalization after 60-minute incubation at *i* 4 °C or *ii* 37 °C

stimulation or inhibition of macropinocytosis did result in a significant increase (3.6 ± 1.3 -fold) or decrease (71.1 ± 11.3 %) in dextran internalization, respectively, relative to dextran internalization in the absence of EGF and EIPA. These observations indicate that at least some fraction of SPACE™ peptide is internalized by non-macropinocytosis pathways at higher concentrations (mg/mL).

Studies Using Endocytosis Inhibitors

To gain additional insight into how SPACE™ peptide mediates internalization and cytoplasmic localization, the effects of chemical inhibitors of internalization were analyzed (Figs. 6a and 7a). Inhibition of caveolin-dependent endocytosis with nystatin had no significant effect on SPACE™ peptide internalization (Fig. 6a). Additionally, there was no observable

Fig. 4 SPACE™ peptide co-localized with dextran in punctate structures, however, extensive diffuse staining of SPACE™ peptide, absent of dextran, was also observed. **a** Representative images of HEK293 cells incubated with *i* media only (control), *ii* TAMRA-dextran (red), *iii* FITC-SPACE™ peptide (green), or *iv* TAMRA-dextran and FITC-SPACE™ peptide. Nuclei are stained with Hoechst 33342 (blue). Arrows indicate co-localization (yellow). **b** Internalization of *i* TAMRA-dextran and *ii* FITC-SPACE™ peptide was quantified with and without co-incubation. Error bars represent mean + SD for $n=3$. $*p<0.05$

Fig. 5 Incubation with SPACETM peptide does not show actin rearrangement characteristic of macropinocytosis stimulation. **a** Representative images of actin (red) after incubation with (i) media only (control), (ii) dextran, (iii) SPACETM peptide, (iv) EGF (macropinocytosis stimulant), and (v) EIPA (macropinocytosis inhibitor). Arrows indicate membrane ruffling characteristic of macropinocytosis stimulation. **b** Internalization of (i) TAMRA-dextran or (ii) FITC-SPACETM peptide incubated with a macropinocytosis stimulant (EGF) or inhibitor (EIPA) relative to incubation in media only. Error bars represent mean $\pm$ SD for $n=3$. * $p<0.05$

dependence on actin (inhibited by cytochalasin D) or tubulin (inhibited by nocodazole), as well as no observable dependence on cholesterol (sequestered by M β CD) (Fig. 6a). The effect of these inhibitors was verified using FITC-transferrin as a control (Figs. 6b and 7b). Further, actin and microtubule depolymerization by cytochalasin D and nocodazole, respectively, was confirmed by visualization with confocal microscopy (Fig. 6c). Incubation with inhibitors at the specified concentrations did not result in any noticeable cell toxicity or morphological abnormalities. Moreover, they did not result in any significant membrane perturbation, as verified by a lack of nuclei staining by EtBr.

Dependence on clathrin-mediated endocytosis was most striking. Inhibition of clathrin-mediated endocytosis using a variety of chemical inhibitors resulted in significant increase in SPACETM peptide internalization (Fig. 7a). Inhibition with

chlorpromazine resulted in a 4.9 ± 0.8 -fold increase in SPACETM peptide internalization. Similarly, inhibition with dynasore, genistein, and primaquine resulted in 6.5 ± 0.7 -fold, 4.6 ± 0.5 -fold, and 4.6 ± 1.4 -fold enhancements in SPACETM peptide internalization, respectively. However, inhibition of clathrin-mediated endocytosis, indicated by internalization of FITC-transferrin, did not correlate directly with an increase in SPACETM peptide internalization. Specifically, inhibition with monodansylcadaverine resulted in a significant decrease (92.7 ± 1.5 %) in FITC-transferrin internalization; however, it had no significant effect on SPACETM peptide internalization suggesting the observed pathway is not simply compensatory for inhibition of clathrin-mediated endocytosis. No appreciable toxicity or membrane disruption was observed after incubation with inhibitors.

Fig. 6 Chemical inhibition analysis of SPACE™ peptide internalization pathway. **a** Internalization of FITC-SPACE™ peptide. **b** Internalization of FITC-transferrin (control). Internalization was normalized to FITC-SPACE™ peptide or FITC-transferrin incubated with HEK293 cells in the absence of inhibitor. Error bars represent mean + SD for $n=3$. * $p<0.05$. **c** Representative images of *i* native actin, *ii* actin in the presence of inhibitor, *iii* native tubulin, and *iv* tubulin in the presence of inhibitor

Discussion

Keratinocytes constitute the majority of the epidermis, which is the first viable tissue layer in the skin. Therefore, they are the first living cell-type to interact with drugs applied on the skin and a targeted cell-type for combating skin-related

disease. However, delivering drugs through the skin and into keratinocytes remains a significant challenge due to transport barriers posed by both the stratum corneum (SC) and cell membrane. To overcome these transport barriers, peptides hold great potential due to their simplicity and diversity, and several peptides have been identified that enhance transport

Fig. 7 Chemical inhibition analysis of clathrin-mediated endocytosis. **a** Internalization of FITC-SPACE™ peptide in the presence of inhibitors. Internalization was normalized to FITC-SPACE™ peptide internalization in the absence of inhibitors. **b** Internalization of FITC-transferrin in the presence of inhibitors. Internalization was normalized to FITC-transferrin internalization in the absence of inhibitors. Error bars represent mean + SD for $n=3$. * $p<0.01$

across both the SC and cell membrane including TAT, poly-R, SPACETM peptide, and penetratin [12–15].

Nevertheless, a critical next step towards developing effective peptide-based, topical drug delivery platforms is to better understand how cell-penetrating peptides (CPPs) interact with keratinocytes. Although much effort has been spent over the last few decades trying to decipher the pathways responsible for CPP internalization in a variety of cell-types [27], and better understand the factors that influence those pathways, controversy surrounding the biological mechanisms that govern CPP internalization and intracellular localization remains [28]. Much of this controversy seems to arise from the fact that CPP internalization is highly dependent on the cell-type and concentration, and studies exploring CPP internalization in keratinocytes at concentrations relevant for topical delivery are severely lacking.

Previous studies have shown that topical application of 50 mg/mL SPACETM peptide is required for maximum macromolecule delivery into the skin, and a fraction of applied SPACETM peptide enters the viable epidermis and dermis [16–19]. Accordingly, we sought to explore SPACETM peptide internalization in keratinocytes within this clinically relevant concentration range (~mg/mL). SPACETM peptide was discovered due to its ability to traverse the stratum corneum, and therefore, could be expected to interact uniquely with keratinocytes. In addition, SPACETM peptide is chemically unique compared to most other CPPs due to its neutral charge and relative hydrophilicity (Table 2). Moreover, whereas TAT, poly-R, and penetratin have been shown to be toxic at concentrations required for drug delivery through the skin [29], SPACETM peptide is non-toxic at concentrations as high as 10 mg/mL [19].

First, we studied SPACETM peptide association with the cell membrane. Interestingly, we observed rapid and extensive association with the cell membrane (Fig. 1a). Moreover, association appears to be specific in nature (Fig. 1c). There was no observable difference in internalization when SPACETM peptide was incubated with molar excess heparin sulfate, which has been shown to significantly reduce the internalization efficacy of positively charged CPPs like TAT and poly-R [28, 37, 38]. Further, there was no observable difference in internalization when SPACETM peptide was incubated with molar excess FBS, which has been shown to significantly reduce the internalization efficacy of amphipathic CPPs like penetratin and model amphipathic peptide (MAP) [28]. Together, these results suggest SPACETM peptide may bind specifically to an extracellular, membrane-anchored, or transmembrane protein.

Mass spectrometry revealed 244 potential targets for SPACETM peptide, and a subset of these based on the frequency of identified peptide fragments are given (Table 1). The highest frequency proteins were structural proteins including actin, tubulin, and keratin. This result is consistent with the observations made while studying membrane association and

internalization. In both cases, we observed clear filamentous-like staining suggestive of binding to extracellular as well as intracellular structural proteins (Fig. 1).

The results are consistent with earlier studies indicating association of SPACETM peptide with keratin. Specifically, we recently reported computational studies on association of SPACETM peptide to keratin and experimentally confirmed the binding constant through chromatography [39]. Further, experimental and computational studies also revealed a direct correlation between keratin binding and transport enhancement through the skin [19]. Keratin 1, Keratin 5, and Keratin 14 are the major keratin subtypes present in epidermal keratinocytes [40] and were identified here as potential binding partners of SPACETM peptide. In addition, SPACETM peptide shows extensive sequence homology to a *Staphylococcus epidermidis* surface protein [12]. Bacterial surface proteins are responsible for binding to extracellular matrix proteins during infection, and recently the binding site for a *Staphylococcus aureus* surface protein with Keratin 10 was crystallized [41]. Similar function could be expected from *S. epidermidis* surface proteins as well. Furthermore, antibodies for keratin have been shown to result in similar staining patterns as observed here [42]. Binding to keratin could result in a signaling response leading to keratin turnover or internalization. Cells are known to accept cues from their extracellular environment leading to migration, receptor internalization, differentiation, etc. This may explain why concentrations of milligram/milliliter of unconjugated SPACETM peptide resulted in enhanced internalization and localization of FITC [18].

In contrast to association with the cell membrane, longer kinetics was observed for internalization. The maximum internalized concentration of SPACETM peptide occurs after 15–30 min, which suggests an active internalization mechanism (Fig. 1b). For example, intracellular concentrations of TAT have been shown to equilibrate within a few minutes after incubation for concentration regimes where internalization is dominated by passive diffusion [34]. SPACETM is a small, cyclic peptide, and it was discovered through skin permeation experiments. Thus, it is highly likely to resist proteases during the short incubation times with cells used in this study. The likelihood of eventual enzymatic degradation in lysosomes, however, is not clear. While such potential degradation may impact the long-term fate of the peptide, we hypothesize that it is unlikely to play a role in cell entry.

FITC intensity is known to be dependent on pH and concentration; thus, conclusions should not be reached based on the absolute intensity of fluorescence in cells. Previous experiments have been also performed over a wide range of SPACETM concentration in the range of 0.1–10 mg/ml, and internalization was found to increase with increasing peptide concentration [18]. Therefore, contributions of FITC self-quenching are not expected to have a significant influence on measurements or conclusions in the study.

Table 2 Chemical composition of SPACE™ peptide is unique among majority of cell-penetrating peptides studied to-date

Name	Sequence	pI	Ref.
SPACE™	ACTGSTQHCG	6.72	(12)
PolyArginine (R8)	RRRRRRRR	13.35	(30)
TAT (49-57)	RKKRRQRRR	13.2	(30)
PEP-1	KETWWETWWTEWSQPKKRKV	10.62	(31)
Penetratin	RQIKIWFQNRRMKWKK	12.83	(32)
Transportan	GWTLSAGYLLGKINLKALAALAKKIL	10.87	(32)
VP22	DAATATGRSASRPTERPRAPARSASRPRRPVD	12.6	(33)
Rev (34-50)	TRQARRNRRRRWRERQR	13.1	(30)
Antp(43-58)	RQIKIYFQNRRMKWKK	12.28	(34)
FHV (35-49)	RRRRNRTRRNRRRVR	13.5	(30)
MAP	KLALKLALKALKAAKLKA	11.4	(32)
MTS	AAVALLPAVLLALLP	5.28	(30)
BMV Gag (7–25)	KMTRAQRRAAARRNRWTAR	13.28	(30)
HTLV-II Rex (4–16)	TRRQRTRRARRNR	13.35	(30)
CCMV Gag (7–25)	KLTRAQRRAAARKNKRNTR	13.21	(30)
P22 N (14–30)	NAKTRRHERRRKLAIER	12.51	(30)
MPG	GALFLGFLGAAGSTMGAWSQPKSKRKV	11.93	(32)
VT5	DPKGDPKGVTVTVTVTVTGKGDPKPD	5.78	(35)
EB1	LIRLWSHLIHIWFQNRRLKWKKK	12.83	(36)
pVEC	LLIILRRRIRKQAHASK	12.98	(36)
CADY	GLWRALWRLLRSLWRLLWKA	12.98	(36)
PreS2-TLM	PLSSIFSRIKDG	5.55	(31)
Calcitonin-derived	LGTYTQDFNKFHTFPQTAIGVGAP	6.75	(31)
SynB1	RGGRLSYSRRRFSTSTGRA	12.8	(31)
Human Integrin β 3	VTVLALGALAGVGVG	5.28	(31)

Black, green, blue, and red letters represent polar uncharged, hydrophobic, basic, and acidic amino acids, respectively

Active internalization was confirmed by comparing internalization at 37 °C to internalization at 4 °C. Specifically, internalization was nearly completely eliminated at 4 °C (Fig. 3). Although low temperatures would be expected to result in a decrease in passive diffusion across the cell membrane due to a decrease in lipid fluidization, the extent of transport reduction would be proportional to the change in diffusion coefficient with some additional contribution from changes in cell membrane binding and partitioning into the lipid bilayer. For example, Zaro and Shen showed a 40 % reduction in passive diffusion of oligoarginine into CHO cells following incubation at 4 °C [43]. Our results, on the other hand, show extensive (>95 %) reduction in internalization that cannot be accounted for by physical effects due to decreased temperature. In addition, the reduction is independent of incubation time. Therefore, some energy-dependent process is highly likely involved.

Active internalization suggests occurrence of endocytosis and nearly all previously discovered CPPs have been shown to be internalized by endocytosis, with the most common endocytosis pathway being macropinocytosis. This is a particularly common observation when sub-micromolar CPP concentrations were studied. Furthermore, SPACE™ peptide was already shown to predominantly exploit macropinocytosis at microgram/milliliter concentrations [12], and macropinosomes are known to be leaky [44], which could explain the extensive diffuse cytoplasmic staining observed.

However, SPACE™ peptide does not appear to predominantly exploit macropinocytosis at higher concentrations (mg/mL) studied here. For example, co-incubation with dextran, a marker of macropinocytosis, resulted in significant diffuse staining of SPACE™ peptide while dextran was retained in punctate structures (Fig. 4a). There was some co-localization between SPACE™ peptide and dextran in punctate spots, which suggests that SPACE™ peptide is internalized to some extent by macropinocytosis; however, it does not appear to be the dominating pathway taken by SPACE™ peptide at higher concentrations (mg/mL). SPACE™ peptide could potentially stimulate macropinocytosis and subsequently perturb the macropinosome leading to release into the cytosol; however, we would expect some diffuse staining by dextran as well since the molecular weights of dextran and SPACE™ peptide used in this study were similar. Moreover, the kinetics of internalization is faster than what would be expected for macropinocytosis. Here, intracellular concentration of SPACE™ peptide maximized after 15–30 min, whereas macropinocytosis is typically reported to show maximum internalization upwards of an hour [45]. SPACE™ peptide could be stimulating macropinocytosis leading to faster internalization; however, if that were the case, we would expect a corresponding increase in overall dextran internalization. For example, TAT peptide was shown to induce macropinocytosis [24] and enhance internalization of dextran during co-

incubation [46]. In contrast, an increase was not observed here; instead, a significant decrease was observed, suggesting SPACE™ peptide may actually compete with dextran for internalization by macropinocytosis without stimulating macropinocytosis (Fig. 4b). As additional confirmation that SPACE™ peptide does not stimulate macropinocytosis, actin rearrangement was studied. Actin rearrangement is characteristic of macropinocytosis and various CPPs, and drug carriers have been shown to initiate actin rearrangement during macropinocytosis stimulation [24, 47, 48]. EIPA was used to inhibit macropinocytosis, and EGF was used to activate macropinocytosis. In line with previous observations, SPACE™ peptide does not induce actin rearrangement (Fig. 5a).

To gain further insight into how SPACE™ peptide mediates internalization and cytoplasmic localization, the effects of chemical inhibitors of endocytosis were analyzed. The lack of effects on SPACE™ peptide internalization after inhibition of internal trafficking machinery (Fig. 6a), together with the lack of diffuse staining by dextran during co-incubation with SPACE™ peptide (Fig. 4a), is striking. Dependence on clathrin-mediated endocytosis was most striking. Inhibition of clathrin-mediated endocytosis with a variety of chemical inhibitors resulted in significant *increases* in SPACE™ peptide internalization (Fig. 7a). This suggests the mechanism of SPACE™ peptide entry may be compensatory for clathrin-mediated endocytosis. Alternatively, SPACE™ peptide internalization may compete for resources of other endocytosis pathways in some way. Indeed, this could explain why we observed significant, albeit differing, enhancements in internalization of SPACE™ peptide when various endocytosis pathways were inhibited with EIPA, chlorpromazine, dynasore, genistein, and primaquine, each with their own mechanism of action on various stages of endocytosis pathways [21].

Taken together, our results demonstrate an internalization profile that is quite different from studies with other CPPs. We show nearly instantaneous membrane binding (saturates after ~1 min) and fast internalization relative to endocytosis, but slow internalization relative to passive transport, that saturates after 15–30 min. The data presented here is consistent with earlier findings of association of SPACE™ peptide with keratin. It is possible, however, that another protein present at a lower concentration may also be at play and could not be identified due to limits of fluorescence detection. Assessment of mechanisms in other cell types is also of interest and should be the focus of future studies.

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