Short Technical Report

Peptide Delivery to Tissues via Reversibly Linked Protein Transduction Sequences

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ABSTRACT

The development of peptide-based therapeutics has suffered from challenges associated with delivery to intact tissue. In skin, an array of protein targets resides only tens of micrometers below the surface; however, because of difficulties in traversing the cutaneous barrier, the potential for peptide-based therapeutics remains unrealized. We have developed a general approach for topical peptide delivery into skin using releasable protein transduction sequences to enable peptide transport across tissue boundaries. Upon entry into the cell, the disulfide linkage between the peptide transduction sequences and peptide cargo is cleaved, permitting the dissociation of the highly charged peptide transduction sequences from the active peptide. A prototype cargo peptide, the hemagglutinin (HA) epitope, was conjugated to a hepta-arginine protein transduction sequence via a releasable disulfide linkage. This construct penetrated the skin to deep dermis within 1 h after topical application. Consistent with the dissociation of the protein transduction and cargo sequences, absorbed protein transduction sequences and HA peptides displayed differential intracellular localization. Reversible protein transduction sequence linkage thus represents a non-invasive platform for tissue delivery of intact peptides with no requirement for viral vectors or parenteral injection and may be of broad utility in molecular therapy.

INTRODUCTION

Peptides and peptidomimetics are

capable of targeting proteins of importance in disease by direct physical binding (3). However, the noninvasive delivery of peptide therapeutics has proven difficult (6). The skin represents an attractive site for refining peptide delivery because it is an accessible somatic tissue that is affected by a large number of disorders potentially amenable to peptide intervention (5,14). However, the penetration of topical therapeutics has been hindered by the cutaneous permeability barrier (8). Recently, a promising approach for delivery across tissue barriers and into cells has emerged in the form of protein transduction sequences. Protein transduction sequences are short sequences of amino acids that are derived from proteins such as HIV1 TAT, *Drosophila antennapedia*, and herpes simplex virus VP22 (7,11,13). Protein transduction sequences deliver molecules into cells by an unknown mechanism (7,13). This mechanism appears to be energy-dependent, is highly conserved, and does not result in a general disruption of cell and tissue barriers (9,12,13). Recent work has established that short oligomers of arginine cross biological membranes more efficiently than the TAT or antennapedia sequences and can be used to facilitate the uptake of a variety of attached molecules (1,9,15). Therefore, we chose to examine the capacity of an oligoarginine protein transduction sequence to assist the delivery of peptides into skin tissue.

MATERIALS AND METHODS

Cells and Cell Culture

Primary keratinocytes from normal individuals were obtained from skin biopsies by Stanford IRB-approved protocols. The cells were isolated and

grown in 50% serum-free medium (Invitrogen, Carlsbad, CA, USA) and 50% medium 154 (Cascade Biologics, Portland, OR, USA) as previously described (2).

Animal Studies

BALB/C and Swiss nude mice aged 6–10 weeks were used for studies *in vivo*. At the site of application on BALB/C mice, the hair was trimmed but not shaven to prevent unintended disruption of the cornified layer of the epidermis. Peptide solution in PBS (50 μ L of 1, 10, or 100 mM) was applied for 30 or 60 min before biopsy. All animal studies were performed in quadruplicate on independently treated and analyzed mice, with consistent findings in all cases.

Analysis of Protein Localization and Tissue Ultrastructure

For immunohistochemistry, 5 μ m skin cryosections were fixed and immunostained as described (10) with a rabbit antibody against the 12 amino acid hemagglutinin (HA) epitope #HA11-A (Alpha Diagnostic International, San Antonio, TX, USA). Detection of the biotinylated transporter sequence was accomplished using FITC-conjugated streptavidin SA-5001 (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's protocol.

Peptide Synthesis and Coupling

Peptides were synthesized using a model 433A Automated Peptide Synthesizer (Applied Biosystems, Foster City, CA, USA). Arginine was protected as its 2,2,4,6,7-pentamethylidihydrobenzofuran-5-sulfonyl (Pbf) derivative and histidine and cysteine as their N- and S-triphenylmethyl (Trt) derivatives,

respectively. Peptides were prepared using the standard *FastMoc* protocol on either a 0.1- or 0.25-mmol scale. All biotin peptides were made with an aminocaproic acid spacer moiety on the *N*-terminus, incorporated during synthesis. For disulfide conjugates, 2,2'-dithiobis(5-nitropyridine) (*N*-Pys) (5 equivalent) was dissolved in a minimal amount of acetic acid:water (3:1) over 30 min at room temperature. HS-C-R7-CONH₂ (1 equivalent) was added as a solution in acetic acid:water (3:1; 1 mL), stirred for 16 h, quenched by removal of the solvent in vacuo, and then the residue was reconstituted in an equal volume mixture of water and ethyl acetate. Layers were separated, and the aqueous phase was extracted and concentrated to provide the activated disulfide, *N*-Pys-SS-CR7-CONH₂, which was used directly with no further purification. The identity and purity of the conjugate were established using analytical RP-HPLC.

RESULTS AND DISCUSSION

A biotinylated hepta-arginine protein transduction sequence was linked via a single *N*-terminal cysteine residue to the 12-amino-acid influenza virus HA epitope tag. This strategy was designed to release the cargo peptide upon entry into the target cell, where the linkage would be reduced. This construct allows the simultaneous detection of the protein transduction sequences via streptavidin-FITC and detection of the HA cargo via an anti-HA epitope antibody (Figure 1). A nonreleasable construct was also synthesized in which the leader and cargo sequences were generated as a single linear peptide. In cells treated with the nonreleasable construct, the antibody signals derived from the protein transduction sequences and HA cargo were co-localized in the cytoplasm (Figure 2). In contrast, the cells treated with the releasable construct displayed predominantly nuclear localization of the leader sequence and cytoplasmic distribution of the HA cargo, suggesting intracellular dissociation of the protein transduction sequence from the cargo. These results were consistent for each of the constructs, regardless of the peptide concentration (1, 10, 100 mM) or

the duration of exposure (1–10 min). The treatment of the cells with non-protein transduction sequence-linked biotin or unconjugated HA peptide yielded only background staining (Figure 2). These data confirm the functionality of the dissociable transporter for peptide delivery and release in cells.

To determine if these peptide constructs can facilitate penetration across tissue barriers *in vivo*, we applied them to the skin of BALB/C and nude mice in concentrations of 1, 10, and 100 mM. Following the topical application of both the releasable and nonreleasable constructs, we observed pep-

Figure 1. Diagram of reversibly linked protein transduction sequences and delivery. The elements include a transporter peptide consisting of (i) a hepta-arginine protein transduction sequence, biotinylated for detection with streptavidin-FITC, (ii) the HA epitope tag cargo peptide that can be readily detected using monoclonal antibodies, and (iii) releasable linkage via a disulfide bond that releases the cargo peptide from the protein transduction sequences upon entry into the intracellular reducing environment. Following entry, the dissociated protein transduction sequence localizes in the nucleus, and the cargo peptide diffuses to bind its intracellular target(s). PTS, protein transduction sequence.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

tide entry into the epidermis and dermis of both mouse strains (Figure 3). Absorption was rapid, with penetration through to the deep dermis occurring within 30 min, with only slight increases in staining intensity observed after 60 min. With either the releasable or nonreleasable construct, the peptide uptake is efficient (approaching 100%) in vivo and in vitro, with essentially every cell within the treatment zone exhibiting staining for the protein transduction sequences and cargo (Figure 3). Application of the HA peptide alone failed to penetrate skin and gave only background staining. Similar to our findings in vitro, the nuclear distribution of the protein transduction sequence was evident in tissue treated with the releasable construct while the HA cargo peptide was distributed more diffusely within target cells. Interestingly, tissue treated with identical amounts of nonreleasable construct displayed less detectable protein transduction sequences and HA peptides than tissue treated with the releasable construct, similar to the findings in vitro.

We have described a nondisruptive method to target protein interactions for therapeutic and experimental purposes. Given the highly charged nature of the protein transduction sequence, its obligate partnership with the cargo peptide may interfere with peptide function or elicit unwanted immunoreactivity. Therefore, we sought to circumvent these issues and expand on the capabilities of protein transduction sequences for peptide delivery into intact tissue by synthesizing a releasable amino acid based linkage between the protein transduction sequences and cargo. We have taken advantage of a naturally occurring cysteine residue in the HA epitope tag to incorporate the releasable disulfide linkage between the biotinylated transporter sequence and cargo peptide, thereby permitting intracellular tracking of both the protein transduction sequences and cargo. Similarly, any peptide sequence that contains or could be modified to contain one or more cysteines could be conjugated in the same manner, making this a broadly applicable strategy for direct peptide transduction of target cells in vitro and in vivo. Utilizing similar methods to transport the cyclic peptide cyclosporin

or a protein kinase C agonist, recent reports have demonstrated that cargo peptides can be biologically active following protein transduction sequence-mediated cell entry (1,9). However, protein transduction sequences are incapable of transporting all proteins. Attempts in our laboratory to use protein transduction sequences to topically

transport larger proteins such as the Cre recombinase have been disappointing (Jennifer Zhang, personal communication). This finding suggests that there may be constraints on the size, conformation, charge ratio, or charge distribution of the peptides that can be transported. Further exploration of the boundaries of this technology is re-

Figure 2. Peptide delivery in primary human cells in vitro. Both a disulfide-linked releasable (releasable R7-HA) and a nonreleasable hepta-arginine protein transduction sequence:HA tag fusion (non-releasable R7-HA) were added to media of primary human skin keratinocytes at 1 (shown), 10, and 100 mM for 1 min (shown) or 10 min. HA peptide alone and unconjugated biotin were applied at the same concentrations and intervals to serve as controls for the protein transduction sequence function. Green (FITC) staining indicates the presence of the biotinylated protein transduction sequences, and red (TRITC) staining denotes the HA epitope cargo. Overlaid FITC+TRITC images are shown in the top two rows. Note that the uptake in all cells for both releasable and nonreleasable constructs and the increased signal intensity in the cells treated with releasable R7-HA. Cells treated with biotin or HA peptide alone resemble untreated cells. Note also that the predominantly nuclear localization of the protein transduction sequences in cells treated with releasable R7-HA in contrast to its retention in the cytoplasm in cells treated with the nonreleasable construct. Hoechst 33342 staining (blue) is shown to confirm the presence of cells in the negative control fields (last panels, bottom three rows).

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Figure 3. Cutaneous peptide delivery after topical application in vivo. 1 (shown), 10, and 100 mM concentrations of releasable and nonreleasable R7-HA were applied for 30 min and 60 min (shown) to BALB/C and nude (shown) mouse skin, and the presence of the protein transduction and cargo sequences was analyzed as in Figure 2. Note the strong staining in skin treated with releasable R7-HA. The non-treated controls (bottom panels) display only background signal. The dotted line indicates the position of the basement membrane zone (BMZ) that demarcates the division between the epidermis (upper region) and the dermis (lower region). Scale bar = 50 µm.

quired. Based on the differential localization of the protein transduction sequences and HA components, the release of cargo peptide is rapid, occurring within 1 min in vitro and within 30 min in vivo, with no significant improvement occurring with additional incubation time. However, further studies are needed to define the precise release kinetics for specific cargo in both cells and tissue. Protein transduction sequence nuclear localization is likely due to the presence of cationic arginine residues that are frequently contained within nuclear localization signals while the localization of the cargo peptide will depend on its site of action (4,12). Therefore, reversible protein transduction sequence linkage may represent a widely useful approach to deliver peptides or peptidomimetic sequences expeditiously within intact living tissue with no cumbersome viral vectors or protein fusions.

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