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Unique arginine array improves cytosolic localization of hydrocarbon-stapled peptides

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ABSTRACT

We have previously reported that miniature proteins containing a distinct array of 5 arginine residues on a folded α-helix – a penta-arg motif – traffic with high efficiency from endosomes into the cytosol and nucleus of mammalian cells. Here we evaluate whether a penta-arg motif can improve the intracellular trafficking of an otherwise impermeant hydrocarbon-stapled peptide, SAH-p53-4^{Rho}. We prepared a panel of SAH-p53-4^{Rho} variants containing penta-arg sequences with different spacings and axial arrangement and evaluated their overall uptake (as judged by flow cytometry) and their intracellular access (as determined by fluorescence correlation spectroscopy, FCS). One member of this panel reached the cytosol extremely well, matching the level achieved by SAH-p53-8^{Rho}, a previously reported and highly permeant hydrocarbon-stapled peptide. Notably, we found no relationship between cellular uptake as judged by flow cytometry and cytosolic access as determined by FCS. This result reiterates that overall uptake and endosomal release represent fundamentally different biological processes. To determine cytosolic and/or nuclear access, one must measure concentration directly using a quantitative and non-amplified tool such as FCS. As has been observed for highly cell permeant miniature proteins such as ZF5.3, optimal penetration of hydrocarbon-stapled peptides into the cell cytosol results when the penta-arg motif is located within more (as opposed to less) structured regions.

1. Introduction

We have previously reported that miniature proteins[1–10] containing a distinct array of 5 arginine residues on a folded α-helix[11,12] – a penta-arg motif – can traffic with high efficiency into the cytosol of mammalian cells.[13] Molecules that embody this motif, such as ZF5.3 and aPP5.3 (Fig. 1), can reach the cytosol with efficiencies as high as 50%, whereas molecules with alternative arginine arrays possess little or no such activity. We refer to these molecules as cell-penetrating miniature proteins (CPMPs).[14] We further showed that ZF5.3 and aPP5.3 are taken up via endocytosis, and that the penta-arg motif facilitates release of the associated protein from Rab5- and Rab7-positive endosomes into the cytosol.[13] Endocytic vesicles have also been implicated in the passage of supercharged proteins,[15] cyclic peptides,[16,17] and the contents of lipid nanoparticles[18] into the cell interior. Here we identify a penta-arg motif that facilitates the efficient trafficking of an otherwise impermeant hydrocarbon-stapled peptide from endosomes into the cytosol and nucleus of mammalian cells.

One family of peptide mimetics with demonstrated utility as research tools and potential therapeutics are hydrocarbon-stapled peptides (Fig. 2).[19–22] Molecules possessing an all-hydrocarbon staple resist proteolytic degradation[23] and can function as potent inhibitors of protein-protein interactions in cell-based assays and animals.[20,24] Indeed, ALRN-6924, a dual inhibitor of MDM2 and MDMX that is related to the hydrocarbon-stapled peptide ATSP-7041,[25] is being evaluated in clinical trials (ClinicalTrials.gov identifier: NCT02264613). Other classes of macrocyclized peptides include cyclic peptides,[26,27] hydrogen-bond surrogate peptides,[28] perfluoroaryl-cysteine cross-linked peptides,[29] and double-click stapled peptides.[30] Yet despite the utility and promise of these and other macrocyclized peptides and extensive effort,[31–34] the physicochemical features necessary to predictably achieve high cytosolic concentration remain unknown.[21,24,35] Some progress has been made towards determining biophysical parameters that affect overall uptake – the amount of material that reaches any cellular compartment – but there have been no quantitative assessments of how much material actually reaches the cytosol and/or

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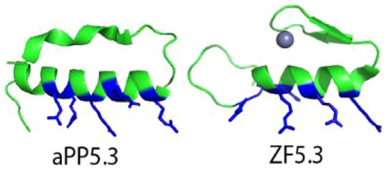

Fig. 1. Cell-penetrating miniature proteins (CPMP) that embody a penta-arg motif. aPP5.3 and ZF5.3 both traffic with exceptionally high efficiency into the cytosol of mammalian cells. The arginine residues that comprise the penta-arg motif are shown in blue.

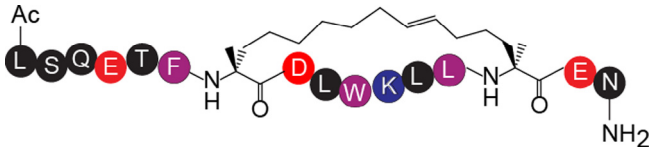

Fig. 2. SAH-p53-4^{Ac}, an example of a hydrocarbon-stapled peptide. See reference [35].

nucleus. Here, we provide this quantitative detail, and identify a distinct penta-arg motif that enhances the intracellular access of an otherwise impermeant hydrocarbon-stapled peptide.

2. Results and discussion

2.1. Hydrocarbon-stapled peptides that embody a canonical penta-arg motif

First, we asked whether the canonical penta-arg motif present in aPP5.3 and ZF5.3 could increase the cytosolic access of an otherwise impermeant hydrocarbon-stapled peptide. For these investigations we began with SAH-p53-4, originally reported by Bernal et al. (Fig. 3A).[35] SAH-p53-4 possesses high affinity for hDM2$_{17–125}$ (K_D = 0.92 ± 0.11 nM), a fragment of hDM2 that contains the p53 binding cleft, but, as judged by flow cytometry, fails to effectively breach the plasma membrane to reach endosomal compartments or the cytosol.[35] To evaluate whether the intracellular access of SAH-p53-4 could be improved with a canonical penta-arg motif, we designed BP1.1 (Fig. 3A). BP1.1 is a variant of SAH-p53-4 with arginine substitutions at residues *i*, *i* + 4, *i* + 7, *i* + 10, and *i* + 11 (residues 14, 18, 21, 24, and 25). This arginine arrangement matches the penta-arg motif in aPP5.3 and ZF5.3. The location of this penta-arg motif within the SAH-p53-4 sequence was chosen to minimize interactions with hDM2-interacting side chains at positions 19, 23, and 26, and with the olefinic residues at positions 20 and 27. BP-1.1^{Rho}, tagged on the N-terminus with Lissamine rhodamine B, was synthesized following a modified version of the protocol reported by Bernal et al.; the purity of the final product was verified by analytical HPLC and the identity was confirmed by LC/MS (Supplementary Fig. 1 and Supplemental Table 1). Two previously reported molecules,[35] SAH-p53-4^{Rho} and SAH-p53-8^{Rho}, were prepared as negative and positive controls, respectively.

The ability of BP1.1^{Rho} to access the cytosol of HeLa cells was studied using fluorescence correlation spectroscopy (FCS) as described previously.[14] FCS is a unique tool for studying the trafficking of molecules across membranes as it provides a precise and accurate measure of the concentration of a fluorescently tagged molecule within the cytosol and/or the nucleus of a living cell. In contrast to flow cytometry, which measures total cell fluorescence – that is, fluorescence due to molecules within all cellular compartments, or even bound to the plasma membrane – FCS allows one to define with accuracy and precision the amount of material that reaches the cytosol and/or nucleus. For initial experiments, we incubated HeLa cells for 30 min with 500 nM of either SAH-p53-4^{Rho}, SAH-p53-8^{Rho}, or BP1.1^{Rho}, and used FCS to determine the fraction of each that reached the cell interior.

HeLa cells incubated with SAH-p53-4^{Rho}, SAH-p53-8^{Rho}, or BP1.1^{Rho} (500 nM) for 30 min were first washed extensively with buffer and trypsin until no plasma membrane-associated fluorescence could be detected using laser-scanning confocal microscopy. Cells were then placed on a home-built confocal imaging system and scanned along the *x*–*z*-dimensions to identify locations for focal volume placement within the cytosol that avoided regions with high punctate signal (Fig. 3B). The nucleus was effectively avoided by placing the focal volume outside of large elliptical regions devoid of bright puncta near the center of the cell. Individual traces were assessed and averaged prior to fitting to a 3D autocorrelation function containing parameters for anomalous diffusion and background autocorrelation as described previously.[14] Evaluation of the correlation curves revealed that SAH-p53-4^{Rho}, SAH-p53-8^{Rho}, and BP1.1^{Rho} displayed wide variation in the maximal autocorrelation signal, indicating an equally wide variation in the concentration of each molecule in the cytosol. Although SAH-p53-8^{Rho} trafficked efficiently to the cytosol, achieving an intracellular concentration of 260 ± 33 nM, SAH-p53-4^{Rho} and BP1.1^{Rho} did not. The final cytosolic concentrations of these two molecules were 62 ± 8 nM and 69 ± 11 nM, respectively (Fig. 3C). These values correspond to trafficking efficiencies of 51.9% for SAH-p53-8^{Rho} and 12.4% and 13.8% for SAH-p53-4^{Rho} and BP1.1^{Rho}, respectively. These data indicate that incorporating a canonical penta-arg motif within SAH-p53-4^{Rho} does not significantly alter its ability to access the cytosol, in spite of the decrease in overall negative charge that accompanied the change (from −2 to +3). Although incorporating a canonical penta-arg motif within a zinc finger domain led to dramatic improvements in cytosolic access,[13] this process has no significant effect on the trafficking of SAH-p53-4.

2.2. Hydrocarbon-stapled peptides that embody a functional penta-arg motif

While structural studies are scarce, we recognized that the display of arginine side chains on an α-helix could be affected by local, staple-induced changes in helix structure and/or dynamics, especially before the ligand engages its intracellular target. In other words, the arginine side chain distribution presented by aPP5.3 and ZF5.3 may not be identical to the distribution when the same arginine pattern is embedded within SAH-p53-4. If so, we reasoned that a slightly different penta-arg array might be more effective in the context of hydrocarbon-stapled peptides related to SAH-p53-4. To test this idea, we designed a panel of six new penta-arg variants of SAH-p53-4 that differed in both the relative spacing of five arginine residues and their axial arrangement (Fig. 4). To ensure that any differences detected are due only to arginine arrangement and not overall electrostatics, all six new SAH-p53-4 variants carried an identical +4 net charge. To evaluate the role of relative spacing, the five arginine residues were either clustered on one face of the helix (BP1.2, BP1.4, and BP1.6) or dispersed among several faces (BP1.3, BP1.5, and BP1.7). To evaluate the role of axial position, some penta-arg motifs were located near the N-terminus (BP1.2 and BP1.3), others were located near the C-terminus (BP1.6 and BP1.7), and still others were dispersed throughout the peptide (BP1.4 and BP1.5). The relative positions of the five arginines in new SAH-p53-4 variants are depicted by helical wheel diagrams (Fig. 4), in which the blue dots represent arginine residues. As with the case of BP1.1, the *i*, *i* + 7 staple (yellow dots) and the hDM2 binding epitope (magenta dots) were preserved.

The set of Rho-labeled SAH-p53-4 variants, BP1.2^{Rho}-BP1.7^{Rho}, were evaluated first using flow cytometry to assess overall uptake (Fig. 5A). Although the set of molecules examined possessed the