Arginine-rich cell-penetrating peptides – The role of heparan sulfate in uptake

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ABSTRACT SUMMARY
A detailed structure-activity relationship regarding the correlation between the binding characteristics of the interaction between heparan sulfate (HS) and a cell-penetrating peptide (CPP) derived from human lactoferrin (hLF) is described. The number of arginine residues was the major factor determining the binding affinity and internalization of the tested CPPs. Uptake negatively correlated with stoichiometry, a finding which suggests that HS acts as a buffer thereby impeding uptake.

INTRODUCTION
Binding to HS at the cell surface is often considered the first step in the internalization process of cationic CPPs. However, currently little is known how the characteristics of the HS-CPP interaction such as affinity, stoichiometry and clustering are related to uptake efficiency. In contrast to the general idea that HS are important for uptake, there are also studies showing a negative influence of HS binding on internalization. For example, we showed recently that the binding to HS is not the only factor determining efficient uptake: arginine-rich CPPs consisting of D-amino acids internalized less efficiently than their L-amino acid counterparts, despite comparable HS binding constants¹. In this study, we investigated a collection of mutants of a CPP derived from human lactoferrin (hLF) with respect to HS binding and uptake. This CPP requires a disulfide bridge for activity and contains several arginines². Because of the requirement of a cyclic conformation, hLF represents an excellent starting point for analyzing structure-activity relationships that may also depend on the peptide conformation. Several peptide variants were designed by varying the number and position of arginine residues, changing the width of the disulfide bridge and mutating residues which are highly conserved in lactoferrins among various species. The structures of the most relevant variants are shown in Figure 1.

EXPERIMENTAL METHODS
All peptides were purchased from EMC microcollections.
Different cells lines (eg. HeLa cells) were incubated for 30 min at 37°C with the indicated peptide concentrations. Uptake efficiencies were visualized by confocal microscopy and quantified by flow cytometry.

Figure 1: Structures of selected hLF variants. The mutated amino acids are shown next to the original amino acid. In Hcy, the cysteines are replaces by homocysteines. All peptides are N-terminally labeled with fluorescein and the C-termini are amidated.

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injected into peptide solutions; concentrations were adapted for the different hLF variants in order to ensure a high signal-to-noise ratio. Measurements were performed in HBS at 25 °C.

To determine preferences for certain HS structures, NHS-activated CodeLink slides containing synthetic HS/heparin oligosaccharides and 5 kDa natural heparin were prepared as described previously\(^3\), treated with 10 μg/ml of fluorescent peptide and the binding was analyzed with the GenePix Pro 7 software.

**RESULTS AND DISCUSSION**

The mutation of amino acids being highly conserved in lactoferrins did not affect the uptake efficiency significantly (data not shown). Most importantly, when arginines were added to the linear variant (lin +4R), which is normally not internalized at all\(^2\), the uptake could be restored but was still worse compared to the corresponding circular variant (+4R) (Fig. 2).

![Figure 2: Flow cytometry histograms of hLF variants. A,C: 5 μM, B,D: 20 μM.](image)

HS binding studies revealed that the affinity was higher for those peptides containing more arginine residues (+4R, lin+4R) which correlated with a higher uptake efficiency (Fig. 2 AB, 3). Although the broader disulfide bridge (Hcy) did not have an effect on uptake (Fig. 2 C, D), the affinity for HS was significantly decreased (Fig. 3A). Another parameter of interest which can be deduced from ITC measurements is the stoichiometry of binding, i.e. how many peptides were bound to one molecule of HS. Here, the trends were similar to the binding constant: the more arginines were present, the lower the stoichiometry of binding suggesting that fewer molecules were required to achieve charge neutralization (Fig. 3B). Stoichiometry showed the strongest negative correlation with uptake.

![Figure 3: Thermodynamic characteristics of HS-hLF interaction obtained by isothermal titration calorimetry. A: HS affinity, B: stoichiometry.](image)

In order to test for structural determinants of HS itself for the interaction with the hLF variants, microarrays were generated with synthetic HS structures (data not shown). The data suggested that peptides are not recognizing specific structures. Rather, the length of the HS chains was critical for binding to occur. Whereas hLF WT and Hcy bound all structures tested, +4R required longer HS chains which is in agreement with the lower stoichiometry because fewer peptide molecules are available to cover the same length of HS chain.

**CONCLUSION**

In general, introduction of arginine residues and cyclisation improved HS affinity and the internalization efficiency. A strong negative correlation was found between stoichiometry and uptake which suggests that HS acts as a buffer that counteracts peptide uptake.

**REFERENCES**