Biochimica et Biophysica Acta 1828 (2013) 1484–1493

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Different roles of cell surface and exogenous glycosaminoglycans in controlling gene delivery by arginine-rich peptides with varied distribution of arginines

Rangeetha J. Naik, Anindo Chatterjee, Munia Ganguli *

Institute of Genomics and Integrative Biology, Mall Road (near Jubilee Hall), Delhi 110007, India

ARTICLE INFO

Article history:
Received 9 August 2012
Received in revised form 27 December 2012
Accepted 19 February 2013
Available online 26 February 2013

Keywords:
Gene delivery
Arginine peptides
Glycosaminoglycans
Heparin-binding motif
Peptide-DNA complex

ABSTRACT

The role of cell surface and exogenous glycosaminoglycans (GAGs) in DNA delivery by cationic peptides is controlled to a large extent by the peptide chemistry and the nature of its complex with DNA. We have previously shown that complexes formed by arginine homopeptides with DNA adopt a GAG-independent cellular internalization mechanism and show enhanced gene delivery in presence of exogenous GAGs. In contrast, lysine complexes gain cellular entry primarily by a GAG-dependent pathway and are destabilized by exogenous GAGs. The aim of the current study was to elucidate the factors governing the role of cell surface and soluble glycosaminoglycans in DNA delivery by sequences of arginine-rich peptides with altered arginine distributions (compared to homopeptide). Using peptides with clustered arginines which constitute known heparin-binding motifs and a control peptide with arginines alternating with alanines, we show that complexes formed by these peptides do not require cell surface GAGs for cellular uptake and DNA delivery. However, the charge distribution and the spacing of arginine residues affects DNA delivery efficiency of these peptides in presence of soluble GAGs, since these peptides show only a marginal increase in transfection in presence of exogenous GAGs unlike that observed with arginine homopeptides. Our results indicate that presence of arginine by itself drives these peptides to a cell surface GAG-independent route of entry to efficiently deliver functional DNA into cells *in vitro*. However, the inherent stability of the complexes differ when the distribution of arginines in the peptides is altered, thereby modulating its interaction with exogenous GAGs.

1. Introduction

Glycosaminoglycans (GAGs) on the plasma membrane are the major contributors of the negative charge on the cell surface. These molecules have been proposed to influence the delivery of cationic gene delivery agents in numerous ways [1]. GAGs can electrostatically bind to cationic gene delivery vectors like lipoplexes, polyplexes and cell penetrating peptides and act as initial attachment sites or receptors to enhance their gene delivery efficiency [1,2]. Alternatively, strong binding to the cell surface GAGs may destabilize the cationic gene delivery vectors [3]. Arginine-containing cell penetrating peptides like the naturally occurring HIV-TAT, Antp, penetratin as well as synthetic arginine homopeptides have been found to interact with cell surface GAGs with high affinity, which promotes their cellular uptake [4–9]. However, when cell penetrating peptides are attached to a cargo either covalently or electrostatically, the interaction of the resulting complexes with the cell surface GAGs may not be similar to that of the free peptides [10–12], as it can be affected by the net charge on the complex, the orientation of the peptides and the availability of free peptides in the complex.

Arginine based peptides have been widely described in the literature as efficient molecular transporters for a variety of cargos, with the potential to transfect a wide range of cells [13–15]. In a previous report, we have demonstrated that oligoarginine, specifically R_{16}, can enter cells independent of the cell surface GAGs, and mediate efficient cargo activity [16]. Although the free peptide exhibits direct translocation for entry, the polyplexes formed by R_{16} complexed with plasmid DNA shows uptake by endocytosis. Both these pathways are also operational in a GAG-deficient cell line [17], supporting the evidence that cell surface GAGs are not essential for cellular entry of arginine homopeptides. Other recent reports have also supported this observation in case of both the free and cargo-conjugated arginine-rich peptide [12,18]. Subrizi et al. have also demonstrated that the cationic charge on the peptides, and not its sequence, determine the efficiency of cellular entry [18]. However, the guanidino group in arginine side chains can interact electrostatically as well as through hydrogen bonds with the sulfate groups on GAGs [19]. This property is exploited by the R_{16}-DNA

Abbreviations: GAG, glycosaminoglycan; CHO, Chinese hamster ovary; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate; EtBr, ethidium bromide; AFM, atomic force microscopy; HS, heparan sulfate; CS, chondroitin sulfate; C6S, chondroitin 6-sulfate

* Corresponding author at: Lab 203, Institute of Genomics and Integrative Biology (CSIR), Mall Road (near Jubilee Hall), Delhi – 110007, India. Tel.: +91 11 27666156, +91 11 27667602; fax: +91 11 27667471.
E-mail addresses: mganguli@igib.res.in, mganguli@igib.in (M. Ganguli).

0005-2736/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2013.02.010

complexes to accommodate low amounts of soluble GAGs (added exogenously) on their surface. The presence of exogenous GAGs protects the complexes leading to minimal destabilization during cellular entry and increased cellular uptake and transfection efficiency [16].

The goal in this study was to check whether the sequence of the arginine peptides is important in determining the interaction of the peptide-DNA complexes with cell surface and soluble GAGs. For this, we designed peptides containing GAG-binding motifs identified by Cardin and Weintraub [20]. These motifs contain clusters of basic charge arranged in patterns of XBBXBX or XBBBXXBX, where B represents a positively charged residue, usually arginine or lysine, and X represents an uncharged or hydrophobic amino acid [20,21]. Control arginine-rich peptide sequences were also chosen which do not contain any GAG-binding motif. This would help us determine whether the charge distribution in the arginine-rich peptides, as listed in Table 1 (with few lysine counterparts for comparison), can modulate their interaction with GAGs and whether the presence of the motifs is favoring a GAG-dependent entry pathway. Our results demonstrate that the presence of arginines is the lone prerequisite for utilization of a GAG-independent route of entry into cells by different arginine-rich peptide-DNA complexes. However, the effect of soluble GAGs on the DNA delivery efficiency of the peptides seems to strongly depend on the charge distribution and spacing of the arginine residues. These results would help in better designing of arginine-rich peptides for delivery in multiple cell types.

2. Materials and methods

2.1. Materials

The peptides used in this study were custom synthesized by G.L.Biochem (Shanghai) Ltd (>95% purity). The plasmids pEGFP-C1, 4.7 Kb (Clontech) and pMIR-REPORT™ Luciferase, 6.47 Kb (Ambion) were amplified in *E.coli* DH5-α and purified using GenElute HP Endotoxin-Free Plasmid MaxiPrep Kit (Sigma). All other chemicals and cell culture media were procured from Sigma, unless mentioned otherwise.

2.2. Cell culture

Parental Chinese Hamster Ovary cells (CHO-K1) were obtained from National Centre for Cell Science Cell Repository, India. The glycosaminoglycan mutant cell lines pgsA-745 and pgsD-677 were obtained from American Type Culture Collection. CHO-K1 and pgsA-745 cell lines were maintained in Ham's F12K medium, and pgsD-677 in Ham's F12 medium. All media were supplemented with 10% (v/v) fetal bovine serum (Life Technologies, U.S.A) and cells were kept in a humidified 5% CO_2, 37 °C incubator.

2.3. Ethidium bromide exclusion assay

20 μl of DNA solution (20 ng/μl) was added to black 96 well plates (Nunc) followed by addition of 20 μl of peptide dilutions at increasing charge ratios. After 10 min, 10 μl EtBr (4.22 ng/μl) was added and further incubated for 5 min in the dark. Fluorescence intensity was measured in DTX 880 Multimode detector (Beckman Coulter) using 535 and 595 excitation and emission filters respectively. The fluorescence of DNA with EtBr was taken as the maximum, i.e. 100% and the relative percentage decrease in fluorescence signal on addition of increasing amounts of peptide was calculated and plotted as percentage of maximum vs. charge ratio.

Table 1
List of peptides used in this study.

Sequences are based on arrays of known heparin binding motifs where B represents basic residue and X represents hydropathic residue.

GAG binding motif	Peptide sequence	No. of residues	No. of positive charges	Acronym
XBBBXXBX	$(ARRRAARA)_4$	32	16	32 A-R
	$(AKKKAAKA)_4$	32	16	32 A-K
	$(ARRRAARA)_3$	24	12	24 A-R
	$(AKKKAAKA)_3$	24	12	24 A-K
XBBXBX	$(ARRARA)_5$	30	15	30 A-R
	$(AKKAKA)_5$	30	15	30 A-K
-	$(RA)_{16}$	32	16	32 RA

2.4. Transfection and luciferase gene expression assay

Polyplexes were prepared, as described previously [16], at different charge ratios with final DNA concentration of 20 ng/μl (pMIR-Report™ Luciferase) and incubated for one hour at room temperature. Indicated amounts of GAGs, expressed as GAG:peptide w/w, were added to the complexes after 30 min of incubation and kept for further 30 min. 100 μl of polyplex (2 μg DNA/well) was added to cells in 24-well plates at 70% confluency in serum free media (OptiMEM, Invitrogen). After 5 hours of incubation at 37 °C, cells were replenished with 500 μl complete growth medium. After 24 hours of transfection, luciferase expression was measured as previously described [16].

For enzymatic removal of cell surface GAGs, CHO-K1 cells were seeded in 24-well plates and treated with GAG lyases after 24 h. Cells were treated with either chondroitinase ABC (250 milliunits) or heparinase III (2 mIU) in 300 μl of digestion buffer (PBS containing 0.1% BSA, 0.2% gelatin and 0.1% glucose) [4] for 1 h at 37 °C. Cells were washed extensively with PBS and serum free media before transfection was carried out as detailed above.

2.5. Flow cytometry analysis

Cells were grown for 24 h in 24-well plates and fluorescently labeled polyplexes at different charge ratios, where DNA was labeled with FITC as described previously [16], were added to cells in serum free media as detailed in the transfection protocol. After 4 h of incubation at 37 °C, cells were washed twice with ice-cold PBS containing 1 mg/ml heparin, and with 0.4% trypan blue in PBS to remove any extracellular fluorescence. Cells were collected by trypsinization, resuspended in PBS and placed on ice. Flow Cytometry measurements were carried out on Guava® EasyCyte™ System (Guava Technologies) using CytoSoft™ software. 10,000 live cells were used for each analysis.

2.6. Stability of polyplexes in presence of GAGs

The polyplexes formed at charge ratio Z (+/−) of 10 (20 μl containing 200 ng DNA) were treated with increasing amounts of GAGs (heparin, heparan sulfate, chondroitin 6-sulfate) and incubated for 30 min before analysis by agarose gel electrophoresis. The amount of the DNA released from the polyplexes was compared with that of the native uncomplexed DNA.

For ethidium bromide (EtBr) intercalation assay, GAGs, at increasing amounts, were added to black 96 well plates (Nunc), followed by addition of 20 μl of polyplex (at Z(+/−) of 10.0) and 10 μl EtBr (4.22 ng/μl) and incubated for 5 min at room temperature in the dark. Fluorescence intensity was measured as mentioned in 2.3. The fluorescence of DNA with EtBr was taken as the maximum, i.e. 100% and the relative percentage increase in fluorescence signal was calculated at increasing concentration of GAGs.

2.7. DNaseI assay

DNaseI assay was performed as previously described [16]. Briefly, the polyplexes at Z(+/−) 10 were incubated with different concentrations of GAGs for 30 min at room temperature. The complexes were then treated with DNaseI (1U) for 30 min at 37 °C. DNaseI