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Mini-review

On the importance of electrostatic interactions between cell penetrating peptides with membranes: A pathway toward tumor cell selectivity?

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ABSTRACT

Cell-penetrating peptides (CPPs) are small molecules of major interest due to their ability to efficiently transport large cargos across cell membranes in a receptor- and energy-independent way and without being cytotoxic to cells. Since their discovery 20 years ago their potential interest in drug delivery and therapeutic diagnosis became undeniable. CPPs are being used to deliver inside cells a large variety of cargos such as proteins, DNA, antibodies, imaging agents and nanoparticle drug carriers including liposomes. Cellular uptake mechanisms of CPPs are still debated and may vary depending on their structure, nature and size of cargo they transport and type of cell line targeted. CPPs are generally rich in positively charged residues, thus they are prone to establish electrostatic interactions with the anionic membrane components (sugars and lipids). Thus understanding the molecular basis of CPP membrane interaction and cellular uptake is crucial to improve their *in vivo* efficiency target-specificity. A great number of studies demonstrated the high potential of CPPs to translocate efficiently therapeutic cargos into cells and some peptides are even in clinical phase studies. Although these molecules seem perfect for a therapeutic or diagnosis purpose, they still possess a small but non negligible drawback: a complete lack of cell type specificity.

Tumor cells have recently been shown to over-express certain glycosaminoglycans at the cell membrane surface and to possess a higher amount of anionic lipids in their outer leaflet than healthy cells. Such molecules confer the cell membrane an enhanced anionic character, property that could be used by CPPs to selectively target these cells. Moreover previous studies demonstrate the importance of electrostatic interactions between basic amino acids in the peptide, especially Arg residues, and the lipid headgroups and glycosaminoglycans in the cell membrane. Electrostatic interactions put at stake in this process might be one of the keys to resolve the puzzle of CPP cell type specificity.

1. Introduction

CPPs have gained much attention these last 20 years since they have a great potential for medical applications. These small molecules can be internalized into cells in a receptor- and energy-independent way and without toxicity to the cells. They can deliver hydrophilic and macromolecular cargos inside eukaryotic cells efficiently without causing significant damage to the cell membrane thus allowing the transport of therapeutic or imaging agents into cells (for a review, see Ref. [1]). The cellular internalization of these peptides has been well studied and proved their efficacy toward a large panel of cells [2]. Although highly efficient in mediating the cellular uptake of different molecules into most cell lines, the use of CPPs appears much more limited to the *in vivo* use mainly because of a complete lack of cell type specificity [3]. Indeed, most of the current anticancer drugs are unable to differentiate between tumoral and healthy cells, leading to systemic toxicity, and thus negative side effects.

The mechanism by which CPPs internalize into cells has been deeply investigated and has given rise to much debate in the literature. Nonetheless a common consensus has emerged and has generally been accepted proposing that multiple mechanisms of

Abbreviations: CPP, cell penetrating peptides; DNA, deoxyribonucleic acid; GAG, glycosaminoglycans; HSPG, heparan sulfate proteoglycans; NP, nanoparticles; SAR, structure/activity relationship.

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cellular internalization intervene. Endocytosis and direct translocation through the membrane can occur depending on the peptide secondary structure, its concentration surrounding the membranes, the type and size of cargo they transport among other properties and experimental conditions [4]. In terms of the cell membrane, selective barrier that the CPPs encounter and need to cross, two families of molecules need to be considered in the understanding of their action mechanism: 1) glycosaminoglycans (GAGs) that have often been shown to be involved in the process of endocytosis among many other different regulating and signaling processes of the cells [5] and 2) lipids whose properties and organization upon peptide interaction have been investigated to shed light into mechanisms of direct translocation of CPPs through the membrane [6–11].

Recent studies have shown that the cell membranes of tumoral and healthy cells differ both in their GAGs and lipid composition and thus such biomarkers could be used to improve CPP selectivity toward cancer cells *vs* healthy ones. The differences in terms of membrane composition between healthy and tumor cells result in an enhanced anionic character for tumoral cell membranes. Considering the important role of electrostatic interactions between positively charged CPPs and the negative charges in the cell membrane, such aspect can be exploited to confer a certain degree of selectivity to CPPs, a property yet lacking for their therapeutic application. Herein we will discuss on the potential of certain CPPs to preferentially bind more anionic membranes rendering them with a "tumor-homing" potential.

2. The cellular membrane: clever customs

Cellular membrane studies are complex due to the high diversity of lipids and proteins present at the cell membrane surface. Cellular membrane is the barrier that protects the cells from external agents but also allows, in a selective manner, molecules to cross them and to be transported to their interior. The membrane is composed of a large variety of lipids, proteins and sugars. Phospholipids are the most abundant molecules in membranes and they play both a structural function and a functional role in regulating and controlling the processes occurring throughout the membrane. GAGs are present in all animal tissues and bind to a large variety of proteins like heparin or growth factors, molecules of the extracellular matrix or molecules implicated in cell adhesion [5,12]. Binding of these proteins triggers multiple and varied functions inside the cells like cell division, angiogenesis, defense mechanisms or endocytosis [13,14]. In the study of the internalization mechanism of CPPs both GAGs and lipids need to be considered to fully understand the system.

Cellular uptake studies at low temperature (4 °C), with a lack of energy (e.g. ATP depletion) or using D-isomer peptides have shown that CPP cellular uptake is both energy- and receptor-independent [15]. However the cellular uptake mechanisms of CPPs depend on a great variety of parameters such as the nature and size of the CPP and its cargo, the nature of the link between the two, the temperature at which internalization experiments are conducted, the cell lines used, among others parameters [16,17]. Direct translocation through the membrane was first evoked as the mechanism of internalization of CPPs, then refuted as an artifact of fixation and later confirmed using fluorescence in living cells [18,19]. This mechanism involves destabilization of the plasma membrane and while endocytosis is inhibited at 4 °C, direct translocation is also decreased because membrane dynamics and fluidity are affected at such low temperature. Thus, assessing direct translocation at low temperatures in living cells leads to an under-estimation of this latter. In fact, to access and study CPP direct translocation through membranes, the use of lipid model systems such as liposomes is ideal and has been widely employed [20]. Direct translocation can occur by different pathways like adaptive translocation, inverted micelle or the pore formation model [7]. Despite a lot of controversy and debate, it is now mainly accepted that both endocytosis and direct translocation through the membrane are implicated in CPP internalization mechanisms [6,7,21]. HSPGs at the cell membrane surface play an important role in these mechanisms.

The presence of HSPGs carboxyl and sulfates moieties strongly contributes to the polyanionic character of cell membranes. They act as an "electrostatic trap" for cationic molecules that are close to the membrane allowing certain of these molecules to penetrate into cells. This joins the finding reported half a century ago that polybasic peptides increase cellular internalization of proteins in culture cells [22]. Moreover it was shown that reticulation of proteins with GAGs increased cellular internalization of CPPs [23]. Many years later it was proven that GAGs deletion at the cell membrane surface decreases or prevents cellular internalization of CPPs [10,24,25]. This demonstrates that CPP internalization capacity depends on the type of interaction established between peptide and membrane lipids rather than the simple presence of positive charged residues in CPPs. Biophysical studies on model systems performed by Seelig and others point to the importance of electrostatic interactions between CPPs and GAGs [10,18,26]. HSPG expression is developmentally regulated and altered in various pathophysiological processes, including cancer. It was observed that GAGs HSPGs are expressed at the healthy cell membrane surface and they were shown to be over expressed at the surface of cancer cells [13,14,27–29]. Indeed the capacity of HSPGs to interact with either soluble ligands or the matrix architecture defines multiple combinations of properties that enable healthy cells to sense and respond to, controlling environmental events. Cancer cells employ various mechanisms to exploit these properties and gain a survival advantage.

For example, the syndecan SDC4 was shown to decrease tumor cell ability to migrate through the regulation of its activator, one of the most expressed growth factor in melanoma cells, the fibroblast growth factor FGF-2 [30]. Concerning the GPI anchored glypicans it has been shown that over-expression of the GPC3 glypican in hepatocellular carcinoma and melanoma induces tumor growth signaling upon binding of its HS chains to Hedgehog and Wnt proteins [31]. Overall, tumor cells have been shown to over-express certain types of proteoglycans such as glypicans and syndecans that are implicated in several aspects of tumorigenesis such as cell adhesion, growth and motility [28,32–34]. The higher abundance of certain types of GAGs in tumoral cells relative to healthy ones could be used to improve CPP selectivity by taking advantage of enhanced electrostatic interactions through their positively charged amino acids.

In what concerns the lipid component in the cell membrane, many studies on model membranes have well characterized and allowed a good understanding of the mode of interaction of CPPs with membranes [35–37]. As per the lipid composition of different cell lines, and especially tumoral *vs* healthy ones, subtle but quite consistent and important differences have been reported. Indeed, during cancer development the lipid composition of the cell membrane is strongly modified and different types of cancer have been associated with unique membrane lipid compositions [38].

Phosphatidylserine (PS), an anionic lipid normally present only in the membrane inner leaflet, has been shown to be important during the process of cells apoptosis [39]. Indeed this lipid acts as a stress signaling at the cell membrane surface and is recognized by phagocytes. This signal acts as an efficient recognition factor as soon as phagocytes are close to the membrane. The PS expressed at the proliferative cells membrane surface is thus a marker for angiogenic blood vessels [40] and is also a receptor of interest for

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