

Chapter 2

Penetratin Story: An Overview

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Abstract

Cell-penetrating peptides are short, often hydrophilic peptides that get access to the intracellular milieu. They have aroused great interest both in academic and applied research. First, cellular internalization of CPPs often involves the crossing of a biological membrane (plasma or vesicular), thus challenging the view of the nonpermeability of these structures to large hydrophilic molecules. Secondly, CPPs can drive the internalization of hydrophilic cargoes into cells, a rate-limiting step in the development of many therapeutic substances. Interestingly, the two mostly used CPPs, TAT and Penetratin peptides, are derived from natural proteins, HIV Tat and Antennapedia homeoprotein, respectively. The identification of the Penetratin peptide, summarized in this review, is intimately linked to the study of its parental natural protein.

Key words: Penetratin, Cell-penetrating peptide, Homeodomain, Homeoprotein

1. Introduction

It is extremely striking that the transgression of the dogma of membrane impermeability to hydrophilic molecules at the origin, 20 years ago, of the cell-permeable peptide field stems from the study of two unrelated transcriptional regulators, HIV Tat protein and Antennapedia homeoprotein. In both cases, the necessity to verify their pure intracellular activity had motivated the addition of these proteins in the extracellular medium. With unexpected results that suggested internalization by cultured cells leading to the development of first cell-permeable peptides, to expression strategies based on direct protein delivery – instead of classical nucleic acid transfection – and to the search for the underlying biological function of protein transduction.

2. The Origin of an Unexpected Observation

In 1988, the capture of HIV-TAT by cells and its transport to the nucleus was described (1, 2). At the same time, our laboratory was trying to correlate neuronal shape and position, and in this position/shape context, had started to investigate the function of homeoprotein transcription factors.

In the mid-1980s, we observed that brain neurons in culture adopt different polarity patterns depending on the origin of the astrocytes on which they were plated (3, 4). It was particularly striking that dendrites would only develop when neurons and astrocytes were derived from the same structure. This allowed us to establish a theoretical link between developmental morphogenetic programs and positional information. At the same time, the homeoprotein family of transcription factors that link organ shape to their positional information was discovered in *Drosophila*. We asked whether morphogenetic programs acting at the multicellular levels might also act at the single cell level on neuronal shape.

Homeoproteins are defined by the nature of their DNA-binding domain, the homeodomain. This domain is highly conserved across homeoproteins and species, and is composed of three α -helices, the third helix being more particularly dedicated to the recognition of the DNA target site (5). We wanted to test our hypothesis by injecting a homeodomain within live neurons. The logic was that the injected homeodomain would gain access to the nucleus and displace endogenous homeoproteins away from their cognate sites, thus revealing their morphological function at the single cell level. We used the homeodomain of Antennapedia for practical reasons and on the basis of the strong sequence conservation between homeodomains. Antagonizing transcriptional activity of endogenous homeoproteins was achieved through the mechanical internalization of FITC-labeled homeodomains into live postmitotic neurons (6–8). The addition of exogenous *Drosophila* Antennapedia homeodomain (AntpHD) induced strong neurite outgrowth as expected that was attributed to a competition between the homeodomain and endogenous homeoproteins for their binding sites (8). But the surprise was total when adding the homeodomain into the culture medium, for a control, we observed the same phenotype. This suggested either that the effect of the injected homeodomain was due to its leakage outside the cells – an artifact – or that the homeodomain was internalized. We verified the latter possibility and observed, much to our surprise, that the 60 amino acid long polypeptide was captured by the cells and addressed to their nuclei (8).

3. Homeodomain Translocation

In an attempt to analyze the neurite promoting function of the homeodomain and its mechanism of action, two different point mutations affecting the specificity of protein/DNA interactions (AntpHD 50A) or the structure of the homeodomain (AntpHD 48S) were introduced (9–11). The DNA-binding capacity of the two mutants is either decreased (AntpHD 50A) or completely abolished (AntpHD 48S), and the biological activity (neurite outgrowth stimulation) is lost in both cases (9–11). Most importantly, translocation into live cells is lost only in the AntpHD 48S mutant, in which a single serine residue replaces three amino acids (tryptophan 48, phenylalanine 49, and glutamine 50). Tryptophan 48 (Trp 48), and phenylalanine 49 (Phe 49) are conserved in almost all homeodomains, and important for the homeodomain structure (10).

This observation was so unexpected and disturbing that we decided to identify the mechanism involved in homeodomain capture. Interestingly, the intracellular distribution showing uniform cytoplasmic staining, and nuclear accumulation was at odd with endocytosis. Indeed, uptake was observed at 4°C, with the same uniform cytoplasmic staining. To preclude that this diffusion was due to AntpHD redistribution following fixation, the same experiments done with a FITC-tagged homeodomain on live cells and with the help of confocal microscopy gave identical results (8). Finally, it was verified that the AntpHD was retrieved, intact, from the cell nuclei at both temperature, demonstrating very limited degradation (12).

4. The Penetratin Peptide

The results with AntpHD 48S suggested the presence of a cell translocation sequence in the third helix. The 16 amino acids of the helix (amino acids 43–58 of the homeodomain) were synthesized, and internalization into live cells was followed thanks to a N-terminal biotin (13). Shorter versions of the same peptide, with N-ter or C-ter deletions, are not internalized suggesting that this sequence, thereafter Penetratin, is necessary and sufficient for internalization.

Similar to AntpHD, Penetratin can be internalized by an energy-independent mechanism at both 4 and 37°C, and has access to the cytoplasm and nucleus from which it is retrieved without apparent degradation (13). Penetratin high content in basic amino acids is reminding of TAT and oligoarginine peptides. In contrast, a unique feature of Penetratin is the presence of

hydrophobic residues, in particular tryptophanes, which are critical to the translocation process (13). Indeed, Penetratin and other basic PTDs differ in their cellular behaviors, even in the same experimental set-up (14–17). Although biophysical and biological studies have greatly help to our comprehension of Penetratin behavior, a full understanding of its mechanism of translocation is still in process.

4.1. Charge and Hydrophobicity: A Dual Mode of Interaction

Because Penetratin composed of D-amino acids (D-Penetratin) and an *inverso* form of the peptide are internalized as efficiently as Penetratin (18), it was concluded that a chiral membrane receptor (usually a protein) is not required for cellular translocation. On the other hand, the specific ability of Penetratin to form multimers in the presence of ionic detergents has led to a close examination of Penetratin/lipid interactions (13). Biophysical studies have established that Penetratin preferentially interacts with anionic phospholipids mainly through electrostatic interactions, followed by limited peptide insertion into the bilayer (19–21). Although the first studies strongly suggested that Penetratin binds to the lipid headgroups, a situation not in favor of direct translocation across pure lipid bilayers (22, 23), a more recent diversification of the experimental models and techniques have revealed a different picture. Penetratin actually crosses pure lipid bilayers, either in the presence of an applied transmembrane pH gradient (24, 25) or in response to a self-generated potential resulting from asymmetric peptide aggregation at one side of the bilayer (electroporation-like mechanism) (19). Recently, the spontaneous insertion of non-aggregated Penetratin in the inner leaflet of lipid bilayers was reported using a novel solid-state NMR technique (26). The absence of Penetratin translocation reported by other groups could reflect an unfavorable lipid composition (27–29).

It must be kept in mind that Penetratin/lipid interaction is a reciprocal process affecting both partners. Penetratin adopts a random coil structure in an aqueous environment but becomes structured in the presence of anionic phospholipids. At a low peptide/lipid ratio (1/325), the peptide adopts an α -helical conformation (13, 30–33). At a high peptide/lipid ratio (1/10), the peptide forms anti-parallel β -sheets (21, 33, 34). Conversely, Penetratin alters the organization of lipid bilayers, and the orientation of lipid acyl chains is modified upon the deep insertion of Penetratin into membrane bicelles (35). When applied on a brain lipid mixture preparation, Penetratin induces the formation of hexagonal phases (31). We have proposed that the transient remodeling of lipid organization induced by Penetratin places the peptide in a pseudo-hydrophilic environment and

allows its transfer from the extracellular medium to the cytoplasm of the cell.

4.2. Influence of Structural Parameters on Peptide Translocation

Mutation analysis has confirmed the contribution of both hydrophobic and electrostatic properties to Penetratin translocation. Mutation of basic residues favors peptide insertion in the acyl chains but destabilizes the bilayer (36). A similar situation is observed upon addition of fluorescent probes to Penetratin, which increases its hydrophobicity (37), and induces a transient destabilization of the plasma membrane in live cells, demonstrated by the uptake of a cell-impermeant DNA dye and the appearance of Phosphatidylserine at the cell surface (38). Taken together many arguments suggest that a subtle interplay between hydrophobic and electrostatic properties of Penetratin is required for its translocation. In fact, even minimal modifications, such as substitution of the two Trp residues by two Phe residues, modify peptide/lipid interactions and impair translocation in live cells (13, 35, 37, 39). By contrast neither peptide helicity nor amphipathicity seems to be required for peptide internalization (18). Indeed, increasing the amphipathicity of Penetratin by mutations increases the toxicity of the peptide rather than its translocation efficiency (40).

4.3. One or More Mechanisms of Penetratin Internalization

Recent studies on the mechanism of internalization of Penetratin in live cells have revealed a more complex picture than previously thought, and concluded to a predominant endocytic uptake and vesicular localization of the peptide (14, 15). This proposal is at odd with a direct translocation process demonstrated by several internalization protocols, in particular at 4°C, and biophysical studies. In fact there is no reason to exclude that Penetratin can be captured by endocytosis depending on cell type and tagging procedure. It remains that, in contrast to Tat, endocytosis is not a prerequisite for Penetratin transfer into the cytoplasm and nucleus. Among possible modifiers of Penetratin uptake are the highly negatively charged carbohydrates that surround most cells, in particular, glycosaminoglycans (GAGs). The complex sugars could restrict Penetratin access to the membrane, promote Penetratin aggregation (41), and induce endocytosis, with the possibility (or not) that the peptide crosses the bilayer latter, once within endosomes. Conversely, GAGs may increase peptide concentration near the membrane and favor its interaction with phospholipids and translocation.

This diversity of mechanisms has been illustrated by several studies (36, 42, 43). For example, the intracellular distribution of internalized Penetratin greatly differ between HeLa and MC57 cell lines, or the macropinocytosis inhibitor ethylisopropylamide (EIPA) decreases Penetratin uptake added at high (50 μ M) but not at low (10 μ M) concentration (17, 44).

5. The First Applications

Soon after the observation of homeodomain translocation, we have demonstrated the usefulness of this process for the efficient cell delivery, and biological activity of hydrophilic molecules. Both anti-sense oligonucleotides (against the β -amyloid precursor protein) and protein domains (C-terminus domain of rab3a) were efficiently internalized by cells in culture upon fusion to AntpHD (45, 46). The first in vivo application of AntpHD-mediated vectorization was the induction of T-cell responses by a peptide derived from the HLA-cw3 cytotoxic-T-cell epitope (47). It appeared very quickly that the 16 amino acid long peptide Penetratin could substitute advantageously for AntpHD, both for oligopeptide and oligonucleotide delivery (48, 49). Since this time, this vectorization strategy has expanded dramatically (50), and proven to be highly versatile toward the nature of the transported cargo (from small drugs to nanoparticles) and the biological context (both ex vivo and in vivo). Most importantly, a large panel of peptides has been characterized on the basis of their cell-penetrating behavior although only some of them have been validated with a biological cargo.

6. Conclusions

More than 10 years after the initial reports, one can ask whether CPP-based cellular delivery has reached maturity. The naïve view of a universal magic CPP bullet that delivers any hydrophilic molecule into the cell has been replaced by a more complex picture, where, for instance, the nature of the transported cargo, its mode of linkage to the CPP, or the targeted intracellular compartment have to be considered. Our knowledge in this field still remains largely empirical rather than predictive, and often relies on the setting up of dedicated experimental protocols, such as those described in this book.

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