CELL-PENETRATING PEPTIDES

Processes and Applications

Ülo Langel

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Preface

In the past 7 to 8 years, a new research field has emerged based on the finding that several short peptides, called cell-penetrating peptides or CPPs, are able to translocate across plasma membranes in a nonreceptor-mediated fashion.

The field started with discoveries in 1988 that the HIV-coded Tat regulatory protein could be taken up by cells when added to culture media,[1,2] and in 1991 that the homeodomain of Antennapedia (a *Drosophila* homeoprotein) was internalized by cells in a receptor-independent manner.[3] In an attempt to better understand the mechanism of internalization, the Antennapedia homeodomain was studied by site-directed mutagenesis; in 1994 it was found that the third helix (amino acids 43 to 58) of the homeodomain of Antennapedia is necessary and sufficient for translocation. This resulted in development of pAntp(43–58) (later called penetratin) and its variants by Professor Alain Prochiantz' group.[4] Shorter Tat-derived sequences with cell-penetrating properties were introduced in parallel.[5]

The discovery that short peptides are able to penetrate into living cells is especially exciting because, today, the CPPs have been shown to provide a new method for cellular delivery of a wide array of molecular cargos.

Biological cell membranes define the cell as a compartment with respect to ions and small and large molecules, including peptides, proteins, and oligonucleotides. Gradients of ions and metabolites are built up across the plasma membranes; special transport proteins, such as carriers, pumps, and gated ion channels, regulate import and export of substances across the plasma membrane together with endocytotic processes. Most of these conventional intracellular delivery processes are ATP-dependent and can be saturated. They suffer from strong limitations in terms of the chemical variability of transported molecules and delivery capacity because the number of the carriers is fixed in the cell and can vary only within narrow limits.

The relative inefficiency and cytotoxicity of modern synthetic DNA delivery systems has been one of the driving forces behind the development of novel cellular delivery vectors. The need to import plasmids and antisense oligonucleotides into cells in sufficient amounts led to the use of nonphysiological and possibly traumatic approaches such as electroporation and lipofection. In addition to being relatively harmful and nonspecific, the latter approaches do not allow targeting to individual cell types. Therefore, development of novel, highly efficient delivery systems applicable to basic and clinical research is highly desirable.

CPPs hold promise of becoming complementary among endogenous transporters, viral transfection, lipofection, and electroporation. CPPs are gentler; the variation of their sequence and inclusion of intracellular addresses may assist their selectivity in terms of delivery.

This handbook is the first effort to collect information available about CPPs. It is divided into three sections: classes of CPPs (Section I), possible mechanisms of action of CPPs (Section II), and applications of CPPs (Section III). The contributors

to the handbook are prominent researchers in the field of CPPs who, together, have been involved in all aspects of CPP development, from discovery of CPPs to their biomedical applications. I acknowledge every contributor for his or her excellent work.

Classes of cell-penetrating peptides

Several families of CPPs are known today, mostly derived from naturally occurring proteins by making use of different *ad hoc* principles in choice of sequences. The original sequences of such CPP families are represented below. These particular sequences fall within some criteria for CPP definition, i.e., they consist of less than 30 amino acids, their cellular internalization is seemingly receptor- or protein-independent and occurs at 4°C, and they have been applied for cellular delivery of cargoes.

Examples of CPPs introduced by different research groups

1. Penetratin[4]	RQIKIWFQNRRMKWKK
2. Tat fragment (48–60)[5]	GRKKRRQRRRPPQC
3. Signal sequence-based peptides[6]	GALFLGWLGAAGSTMGAWSQPKKKRKV
4. pVEC[8]	LLIILRRRIRKQAHAHSK
5. Transportan[9]	GWTLNSAGYLLKINLKALAALAKKIL
6. Amphiphilic model peptide[10]	KLALKLALKALKAALKLA
7. Arg_9[11]	RRRRRRRRR

Sequences 1 to 4 are derived from naturally occurring proteins; sequences 5 to 7 represent artificial synthetic or chimeric peptides. Many analogs discussed in the following chapters exist for several of these parent peptides.

CPPs were first derived from naturally occurring proteins like Antennapedia of *Drosophila*, Tat of HIV-1, and VP22 of HSV (protein transduction domains, PTD), followed soon by designed chimeric peptides like MTS–NLS and transportan, model peptides, and other PTDs.[7,12,13] Numerous analogs of the above-mentioned CPPs have been designed and synthesized, giving rise to families of respective peptides. Both classes of CPPs, whether protein-derived and designed or combined, have been vastly expanded by new peptides and derivatives. The motifs responsible for penetration are generalized, at least for some CPPs, and this information has fueled design and successful application of poly-arginine-mimicking peptoids.[14] Examples of these CPPs are described in detail in Chapters 1 through 7.

Although these chapters cover most described CPPs today, some constantly emerging CPPs or, more exactly, novel vector developments are not included in this handbook. These include the lipophilization approach,[15-17] design of 7TM receptor antagonists,[18] and, possibly, some very recent developments that have not been tested carefully yet.

Mechanisms of cell penetration

Among the many short peptides applied in drug delivery, the choice of CPPs presented in this book is somehow arbitrary since the term *receptor* or *protein-independent* uptake is complicated to define. For simplification, internalization "at 4°C" has been included in the definition. Clearly, further studies on CPP uptake mechanisms are necessary before a consensus definition of CPPs can be proposed. In Chapters 8 through 14 possible mechanisms of cellular uptake of CPPs are discussed.

We have to admit that today, despite numerous studies carried out in the field, the mechanism of uptake of CPPs is still not clarified. In addition, it is likely that mechanisms may differ between distinct CPPs.

However, we feel that the CPPs shown earlier differ from other classes of peptides used in drug delivery, in particular peptides recognized by cell-surface receptors, because they are captured by cells in absence of a chiral receptor, or even when endocytosis has been blocked. This difference has a very high impact in cargo delivery, since CPPs and their cargoes do not accumulate in endosomes and lysosomes and thus have better access in the same time to other compartments less sensitive to degradation. The uptake mechanisms of CPPs also differ from those of a wide variety of toxins and antimicrobial peptides, as they do not form pores within the membranes. Therefore, this handbook describes the latter class of membrane-active peptides only briefly. Chapter 14 has signal sequences as its subject due to possible mechanistic similarities between the uptake of these peptides and that of CPPs.

Attempts have been made to extract structural information that would predict the uptake process of the transport peptides. Positive charges in side chains of Lys/Arg, amphiphilicity of the peptide, presence of Trp or Phe residues in certain positions, and length of the polypeptide chain have been proposed as crucial factors determining cellular uptake. However, general rules for cellular uptake of peptides are only beginning to emerge. Therefore, rational design of novel transport peptides, sequence comparison, SAR studies, molecular modeling, biophysical characterization, and multivariate analysis will be necessary for successful application of CPPS in research and therapy. These topics are discussed throughout the handbook, particularly in Section II.

Applications of CPPs

Traditionally, polypeptides and oligonucleotides are considered of limited therapeutical value because of their low biomembrane permeability and their relatively rapid degradation. This is an obstacle to their use in biomedical research and as pharmaceutical substances. Indeed, the possibility to manipulate intracellular biological targets would increase if large-sized hydrophilic compounds could be addressed intracellularly, without severe limitation on amounts inherent to the necessity to cross lipid bilayers. Thus, the discovery that CPPs translocate across the plasma membrane of live cells and permit intracellular transport of cargoes, such as conjugated peptides, proteins, oligonucleotides, λ phages, and nanoparticles has opened new possibilities in biomedical research and therapy. In Section III, several such applications are covered, including methods for CPP conjugation and microbial applications.

In conclusion, a constantly growing number of cell-penetrating peptides are available today and have been applied for cellular delivery of a variety of bioactive cargoes with seemingly no strict size limit. This handbook summarizes that which has already been achieved. Although the mechanisms of cellular uptake of CPPs are not yet clarified, attempts have been and are being made to help solve this question, thus opening the way to rational design of peptidic and nonpeptidic carriers. Clinical applications will then be within reach.

Ülo Langel

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Editor

Ülo Langel is a professor at the department of neurochemistry and neurotoxicology, Stockholm University, Sweden. Dr. Langel graduated from Tartu University, Tartu, Estonia, as a bioorganic chemist in 1974; he has received his Ph.D. twice: in 1980 from Tartu University (bioorganic chemistry), and in 1993 from Tartu University and Stockholm University (biochemistry and neurochemistry). His professional experience includes a career at Tartu University (from junior research fellow to associate professor and visiting professor, from 1974 to the present); The Scripps Research Institute, La Jolla, California (associate professor, 2000 to 2001); and Stockholm University (from research fellow to associate professor and professor, from 1987 to the present).

Professor Langel has been selected as a fellow member of the International Neuropeptide Society (1995), and is also a member of the International Society for Neurochemistry, European Peptide Society, Swedish Biochemical Society, and Estonian Biochemical Society. He has been awarded a White Star Order, 4th class, by Estonian Republic. Dr. Langel has been an invited lecturer at numerous international conferences and is co-author of more than 140 scientific articles and 5 patents.

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Contents