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Review

Arginine-rich peptides: potential for intracellular delivery of macromolecules and the mystery of the translocation mechanisms

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Abstract

A basic peptide derived from the human immunodeficiency virus (HIV)-1 Tat has been reported to have the ability to translocate through the cell membranes and to bring exogenous proteins into the cells. We have demonstrated that these features were observable among many arginine-rich peptides including those having a branched chain structure. Based on these findings, the presence of a ubiquitous internalization mechanism for the arginine-rich peptides has been suggested. In this review, the potential of these peptides for the intracellular delivery of macromolecules and the mystery of the translocation mechanisms are reviewed.

Keywords: Arginine-rich peptides; Macromolecules; Translocation mechanisms

1. Introduction

Basic peptide mediated protein delivery into cells has been attracting much attention as a novel technology for the efficient incorporation of various peptides and proteins into living cells. Human immunodeficiency virus (HIV)-1 Tat-(48–60) (Fawell et al., 1994; Vivès et al., 1997) and *Drosophila* Antennapedia (Antp)-(43–58) (penetratin) (Derossi et al., 1994) are the well-known peptides to have these activities. Using the Tat peptide, even a protein with molecular weight of 120 kDa (β-galactosidase) has been reported to be delivered in its active form to various organs when intraperitoneally administrated to mice (Schwarze et al., 1999). Not only proteins but also various macromolecules such as oligoDNAs (Astriab-Fisher et al., 2000), magnet beads with a diameter of ~50 nm (Josephson et al., 1999; Lewin et al., 2000; Wunderbaldinger et al., 2002), chelate molecules bearing radioisotopes (Polyakov et al., 2000), and even liposomes with a diameter of ~200 nm (Torchilin et al., 2001) have been reported to be successfully incorporated into cells (Table 1). Modulation trials of cellular function have also been reported. Dowdy and his coworkers prepared various fusion proteins of Tat with bioactive proteins, and induced the regulation of cellular events such as the induction of apoptosis and the modulation of cell cycles (Table 2).

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Table 1
Intracellular delivery of various molecules using basic carrier peptides

Molecules	Carrier peptides	Conjugation
Synthetic peptides	Antp, Tat, oligoarginine	Direct attachment, disulfide
Proteins	Tat, oligoarginine, Antp	Chemical cross-link, genetic
Magnet beads	Tat	Chemical cross-link
Liposomes	Tat	Chemical cross-link
Antisense oligoDNA	Antp, Tat	Disulfide
Radioisotopes	Tat	Chelate
Natural products	Oligoarginine	Chemical cross-link

Table 2
Modulation of cellular function by the incorporation of exogenous peptides and proteins

Cargo molecule	Biological response	Literature
Proteins		
$p16^{INK4a}$ (Cdk4/6 inhibitor)	G_1 arrest	Ezhevsky et al., 1997, 2001
$p14^{INK4a}$ (Cdk4/6 inhibitor)	G_1 arrest	Kato et al., 1998
$p27^{Kip1}$ (Cdk2 inhibitor)	Cell migration	Nagahara et al., 1998
Caspase-3	Apoptosis	Vocero-Akbani et al., 1999
IκBα mutant	Inhibition of osteoclastogenesis	Abu-Amer et al., 2001
Peptides		
Bak BH3 domain peptide	Apoptosis	Holinger et al., 1999
cGPK-Iα inhibitory peptide	Inhibition of vasodilation	Dostmann et al., 2000
IKKβ C-terminal peptide	Inhibition of NF-κB activation	May et al., 2000
εPKC-derived peptide	Reduction of ischemia	Chen et al., 2001a,b
PKA inhibitory peptide	Inhibition of long-lasting LTP	Matsushita et al., 2001
MEK1 N-terminal peptide	Inhibition of MAPK activation	Kelemen et al., 2002

Abbreviations: Cdk, cyclin-dependent kinase; BH, Bcl-2 homology; cGPK, cGMP-dependent protein kinase; IKK, IκB-kinase; PKC; protein kinase C; PKA; protein kinase A; LTP; long-term potentiation; MEK; MAPK/ERK kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.

They also reported that the administration of urea-denatured proteins gave better transduction efficiencies than when administrated in their naturally folded structure (Nagahara et al., 1998). These results suggested that the delivered proteins reformed their active structures in the cells. Cellular events were also modulated by synthetic peptides conjugated with basic peptides including the Tat and Antp peptides. The incorporated peptides modulate the activities of various cellular kinases and proteases that are important for cellular function (Table 2). Another fascinating example is the incorporation of cyclosporin A through the skin (Rothbard et al., 2000). Using a heptaarginine segment, cyclosporin A was efficiently delivered into dermal T lymphocytes, which inhibited inflammation. In contrast to the significant potential of these approaches, the internalization mechanisms of these peptides and their conjugates are still unclear.

2. Internalization of various arginine-rich peptides

The sequence of Tat-(48–60) that is critical for the translocation, GRKKRRQRRRPPQ, is highly basic, containing six arginines and two lysines in 13 amino acid residues (Vivès et al., 1997). Antp-(43–58) is also a basic peptide having three arginines and four lysines. Based on our expectation, the membranes are of a hydrophobic condition and these highly basic and hydrophilic peptides should not go through the membranes. However, in fact, these peptides quite easily go through the membranes. It was reported that the Tat peptide entered the cultured cells and reached the nucleus in a few minutes. What is more interesting is that the internalization was not caused by the typical endocytosis; the internalization of these peptides is not crucially suppressed at 4 °C where the typical endocytosis pathway should significantly be suppressed (Derossi et al., 1994; Vivès et al., 1997). Endocytosis inhibitors were also ineffective for the internalization of the Tat peptide (Polyakov et al., 2000). My first impression of these peptides was to wonder why such basic peptides could go through the membranes so easily and if these were only peptides

going through the membranes. To answer these questions, we have started the following study on the membrane permeable basic peptides.

What we first examined was if the D-amino acid substituted Tat and arginine-substituted Tat (residues 49–57 of the Tat peptide has been substituted for arginine) could go through the membrane (Fig. 1) (Futaki et al., 2001). If there is a specific receptor or transporter for the Tat peptide, internalization of these peptides should not be as efficient as the Tat peptide. Based on fluorescence microscopic observation of the fluorescein-labeled peptides, little difference in the internalization efficiency and cellular localization was observed among these peptides. Whereas little internalization was observed for the lysine-rich peptides, the nuclear localization signal (NLS) peptides from simian virus 40 (SV40) (PKKKRKV) and that from nucleoplasmin (KRPAAIKKAGQAKKKK). These results suggested that the arginines played an important role in the translocation rather than the lysines. Arginine-rich segments are often seen among various RNA and DNA binding peptides. We have shown that many peptide segments are translocated through the cell membranes as efficiently as the Tat peptide and were localized both in the cytosol and nucleus as was the case of the Tat peptide. These peptides included the RNA-binding segment derived from HIV-1 Rev, flock house virus (FHV) coat, brome mosaic virus (BMV) Gag, and human T-cell lymphotrophic virus (HTLV)-II Rex proteins. These peptides contained more than seven residues of arginine (Fig. 1). A lesser degree of internalization was observed for the peptides containing the smaller number of arginine residues in the sequences. These results also suggested the importance of arginines in the internalization. We also examined the DNA binding peptides derived from the leucine zipper proteins. The peptides derived from cFos, cJun and the GCN4 transcription regulator proteins turned out to have the ability of internalization.

(a) Tat, Antennapedia and the related peptides

HIV-1 Tat-(48-60)	GRKKRRQRRRPPQ
D-Tat	G*rkkrrqrrrppq*
R_9-Tat	GRRRRRRRRRPPQ
Antennapedia-(43-58)	RQIKIWFQNRRMKWKK

(b) RNA-binding peptides

HIV-1 Rev-(34-50)	TRQARRNRRRRWRERQR
FHV coat-(35-49)	RRRRNRTRRNRRRVR
BMV Gag-(7-25)	KMTRAQRRAAARRNRWTAR
HTLV-II Rex-(4-16)	TRRQRTRRARRNR

(c) DNA-binding peptides

Human cFos-(139-164)	KRRIRRERNKMAAAKSRNRRRELTDT
Human cJun-(252-279)	RIKAERKRMRNRIAASKSRKRKLERIAR
Yeast GCN4-(231-252)	KRARNTEAARRSRARKLQRMKQ

(d) Oligoarginine

R_8	RRRRRRRR

Fig. 1. Examples of membrane-permeable basic peptides. D-amino acids are denoted in italics.

3. Importance of arginine for translocation and the optimum number for the internalization

Because many basic peptides, which do not have amino acid sequences in common, can go through the membranes, we examined if these peptides have a similar secondary structure. Their CD spectra in methanol did not show the peptides sharing a typical secondary structure; the Tat peptide showed a random structure and the Rev peptide gave a helical structure.

To further study the roles of arginines during translocation, we synthesized arginine peptides with various chain lengths (R_n: $n=4$–16) and examined the translocation efficiency and cellular localization of these peptides. The R_4 (tetramer of arginine) did not show significant internalization. The R_6 (hexamer) and R_8 (octamer) showed a high efficiency of internalization and localization of the peptides in the nucleus was also observed. However, as the chain length further increased, the efficiency of the translocation decreased again. In the case of R_{12} (12mer) and R_{16} (16mer), the peptides seemed to reside on the membranes and significant localization of the peptides in the nucleus was not recognized. The chain-length dependency was also observed by the quantification of the internalized oligoarginines (Mitchell et al., 2000; Suzuki et al., 2002).

Wender et al. (2000) have pointed out that even a peptoid (*N*-alkyl-glycine) oligomer bearing multiple guanidino side chains has the translocation ability. Recently, it was also reported oligomers of

β-amino acids bearing guanidino moieties translocated through the cell membranes (Umezawa et al., 2002). These findings suggested that the guanidino function in arginine played a very important role in the translocation. The guanidino moiety has been reported to form an ideal hydrogen bonding structure with the phosphate backbones of RNA (Calnan et al., 1991). Similar hydrogen bonding can be formed with the phospholipids in the lipid bilayers. Whether such a hydrogen bonding structure has something to do with the translocation of the peptides or only the highly basic structure of the guanidino function ($pK_a \sim 12$) is necessary is not clear at this time.

We then examined whether a linear structure was necessary for the translocation. We synthesized four-branched chain peptides with arginine residues on the N-terminus of each branched chain. Here $(R_2)_4$ (total number of arginine = 8) (Fig. 2) also showed efficient translocation as was seen in the linear R_8 peptide. When the number of arginine residues in the branched chain peptides increased or decreased, the efficiency of the translocation decreased. Thus the necessity of a linear structure was not recognized and the cluster of arginine was suggested to be important for translocation. What was more interesting, other branched chain peptides such as $(RG_3R)_4$ and $(R_1)_8$ (Fig. 2), which also have eight residues of arginine, showed different spectra of cellular localization (Futaki et al., 2002). When the peptides were cross-linked with a model protein

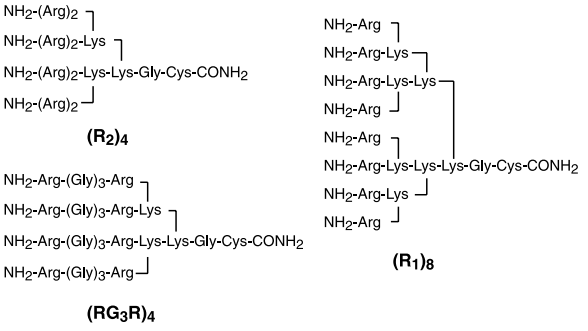

Fig. 2. Branched-chain arginine peptides showing a different cellular localization.

(carbonic anhydrase, 29 kDa), the conjugate was delivered into the cells and localization of the protein in the cell was different from each other. These results suggested that further refinement of the peptide design might lead to the development of organelle-specific delivery peptides.

4. Common features shared by the arginine-rich peptides

The HIV-1 Tat peptide has unique characteristics of internalization together with its carrier ability to bring exogenous proteins into cells. The peptide was internalized and reached the nucleus within 5 min. The internalization of the peptide was not abolished even at 4 °C, where the typical endocytic pathway should suffer a considerable suppression (Vivès et al., 1997). The toxicity of the peptide was quite low compared with other membrane permeable peptides. Do these basic peptides also share similar characteristics in translocation? Together with the branched chain peptides, the RNA-binding peptides and oligoarginine such as R_8 have a carrier ability to bring an exogenous protein into the cells (Futaki et al., 2001). The internalization of the Rev peptide and R_8 peptides was not abolished nor affected in the presence of various endocytosis inhibitors such as colchicine, wortmannin, and chloroquine, metabolic inhibitors such as sodium azide, and a caveolae inhibitor, nystatin (Suzuki et al., 2002). These peptides were not highly toxic to the cells. As judged by the MTT [= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay, more than 85% of the cells survived for 24 h in the presence of 100 μM of these peptides. The effect of these peptides for membrane integrity was examined using the lactate dehydrogenase (LDH) release assay. Mastoparan, a bee venom-derived amphiphilic peptide, released almost 30% of the LDH in the cells. In contrast, the release from the cells treated with 100 μM of the Rev, Tat, and the R_8 peptides was substantially negligible. Thus the damage these peptides gave to the cell membranes was assumed to be relatively small. These three peptides seem to share a common internalization pathway or localization site in the cells. Cells were

then treated with a fluorescein-labeled Tat peptide with an excess amount of the non-labeled Rev peptide. In such a case, the fluorescent intensity from the cells significantly decreased. Using a fluorescein-labeled Rev peptide, similar results were obtained in the presence of the non-labeled Tat peptide. Even in the case of the branched chain $(R_2)_4$ peptide, basically a similar tendency was observed in terms of the fast internalization, uptake at 4 °C, relative low toxicity, and competition with the Tat peptide. These results suggested that there could be common internalization mechanisms among the arginine-rich basic peptides. The precise mechanism of the internalization is not clear at this time. Involvement of cell surface sulfated proteoglycans such as heparan sulfate has been suggested in the internalization pathway. Heparinase III or anti-heparan–sulfate-antibody treatment of the cell lowered the peptide internalization (Suzuki et al., 2002). It would be plausible that these negatively charged proteoglycans would take part in the concentration of the basic peptides on the membranes. On the other hand, internalization of the Tat peptide to the cells defective in heparan sulfate proteoglycan was also reported (Silhol et al., 2002). Further study is necessary to answer whether sulfated proteoglycans including those other than heparan sulfate is indispensable for the internalization of the basic peptides.

5. Mystery of the translocation mechanism

Recently, it has been reported that Tat-linked liposomes with a diameter of 200 nm translocated through the cell membranes while retaining its vesicle structure (Torchilin et al., 2001). From this result, it is deduced that the lipid of the liposomes did not fuse with the cell membranes. On the other hand, it would be necessary that at least a cleft or pore larger than the size of the liposomes opened in the membranes. The size is larger than that of the cellular proteins including LDH and these proteins should have been leaked out if such an opening was induced in the cell membranes. Whereas the damages of these peptides to the cell membranes have been proved not to be very critical nor has significant leakage of LDH not been recognized (Suzuki et al., 2002). Torchilin et al. suggested the possibility of the reverse micelle model proposed in the translocation of the Antp peptide (Derossi et al., 1994; Prochiantz, 2000). They emphasized the importance of the polyethylene glycol (PEG) linker between the liposomes and the Tat peptide. The linker would be useful to prevent the membranes of the liposomes from attaching and fusing to the cell membranes. Thus, although the carrier ability of the Tat and the related peptides is magnificent, the mechanism itself is still in a mystery. We have to note that, even though all of the above peptide-mediated delivery systems show efficient internalization, the possibility of the involvement of more than one internalization mechanisms could not be excluded. Even between the Tat and Antp peptides, there could be a slight difference; the Tat peptide has been employed for the delivery of many proteins whereas the application of the latter peptide has mainly been reported for the delivery of peptides of ~ 30 residues. The Antennapedia peptide shows a helical structure, which was reported to be important for the translocation. However, the helical structure does not seems to be crucial in the case of the Tat peptide. Internalization of the Antennapedia peptide slightly decreased at 4 °C, however, in the case of the Tat peptide, the temperature dependence was quite low. Also, we have to be aware that cargo molecules can have some influence on the pharmacokinetics and delivery of basic peptides, especially in vivo (Lee and Pardridge, 2001).

The fact that many arginine peptides with various structures can translocate through the cell membranes implies the possibility of finding peptides that shows specificity to some organs and organelles in the future. There is another possibility that many peptides having such basic segments can go through the membranes, which may also lead to novel findings such as the mechanisms of viral infection. The understanding of these mechanisms would open new avenues to the establishment of novel delivery systems into the cells.

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