Arginine-rich Peptides
AN ABUNDANT SOURCE OF MEMBRANE-PERMEABLE PEPTIDES HAVING POTENTIAL AS CARRIERS FOR INTRACELLULAR PROTEIN DELIVERY*

Shiroh Futaki‡, Tomoki Suzuki‡, Wakana Ohashi‡, Takeshi Yagami‡, Seigo Tanaka‡, Kunihiro Ueda‡, and Yukio Sugiuira‡

From the ²Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011 and the §National Institute of Health Sciences, Setagaya-ku, Tokyo 158-8501, Japan

A basic peptide derived from human immunodeficiency virus (HIV)-1 Tat protein (positions 48–60) has been reported to have the ability to translocate through the cell membranes and accumulate in the nucleus, the characteristics of which are utilized for the delivery of exogenous proteins into cells. Based on the fluorescence microscopic observations of mouse macrophage RAW264.7 cells, we found that various arginine-rich peptides have a translocation activity very similar to Tat-(48–60). These included such peptides as the p-aminocid- and arginine-substituted Tat-(48–60), the RNA-binding peptides derived from virus proteins, such as HIV-1 Rev, and flock house virus coat proteins, and the DNA binding segments of leucine zipper proteins, such as cancer-related proteins c-Fos and c-Jun, and the yeast transcription factor GCN4. These segments have no specific primary and secondary structures in common except that they have several arginine residues in the sequences. Moreover, these peptides were able to be internalized even at 4 °C. These results strongly suggested the possible existence of a common internalization mechanism ubiquitous to arginine-rich peptides, which is not explained by a typical endocytosis. Using (Arg)n (n = 4–16) peptides, we also demonstrated that there would be an optimal number of arginine residues (n – 8) for the efficient translocation.

Recently, methods have been developed for the delivery of exogenous proteins into living cells with the help of membrane-permeable carrier peptides such as HIV-1 Tat-(48–60) and Antennapedia-(43–58) (1–11). By genetically or chemically hybridizing these carrier peptides, the efficient intracellular delivery of various oligopeptides and proteins was achieved. One of the most amazing examples is the Tat-β-galactosidase fusion protein (4), which has a molecular mass as high as 120 kDa.

Intraperitoneal injection of the protein resulted in delivery of the protein with β-galactosidase activity to various tissues in mice, including the brain. The peptide-mediated approaches would allow the incorporation of peptides containing unnatural amino acids or nonpeptide molecules such as fluorescence probes. These methods would become powerful tools not only for therapeutic purposes as an alternative to gene delivery, but also for the understanding of the mechanisms behind fundamental cellular events, such as signal transduction and gene transcription.

Besides the potential of Tat-(48–60) as a protein carrier, the internalization mechanism of the peptide attracted our interest. For example, Tat-(48–60) (GRKKRRQRPRPQ) is a highly basic and hydrophilic peptide, which contains 6 arginine and 2 lysine residues in its 13 amino acid residues. However, the peptide was reported to be translocated through the cell membranes in 5 min at a concentration of 0.1 μM (2). Internalization of the peptide was not inhibited even at 4 °C. The peptide is less toxic to cells than other basic membrane-interacting agents. The above features suggested that the internalization mechanism of Tat-(48–60) was completely different from the typical transmembrane mechanisms reported so far. Questions arise as to whether such an efficient translocation mechanism is specific for Tat-(48–60) and Antennapedia-(43–58) peptides and what is the mechanism of the highly efficient internalization. Based on experiments using synthetic peptides, we suggest the possibility of a very similar translocation mechanism to Tat-(48–60) present among the various arginine-rich peptides. We also suggest the possible existence of the optimum chain length of arginine peptides for the internalization.

EXPERIMENTAL PROCEDURES
Peptide Synthesis and Fluorescent Labeling—All the peptides used in this study were chemically synthesized by Fmoc (9-fluorenylmethoxycarbonyl)-solid-phase peptide synthesis on a Rink amide resin as reported previously (12). Fluorescent labeling of the peptides was conducted by the treatment with 1.5 eq of 5-maleimidofluorescein diacetate (Sigma) in dimethylformamide-methanol (1:2) for 3 h followed by reverse-phase HPLC purification. The fidelity of the products was ascertained by time-of-flight mass spectrometry.

Conjugation of Carbonic Anhydrase with Basic Peptides—Carbonic anhydrase in phosphate-buffered saline (PBS) was simultaneously treated with fluorescein-5(6)-carboxamidocaproic acid N-hydroxysuccinimide ester (Sigma) and N’-6-maleimidocaproyloxy)succinimide ester (Dojin) (15 eq, each) at roomtemperature for 1 h to introduce the fluorescein and the maleimide function to the protein. After the removal of the unreacted reagents by gel-filtration on a Sephadex G-25 (Amer- sham Pharmacia Biotech) column, the cysteine of the respective arginine-rich peptides was allowed to react with the maleimide moiety on the above fluorescein-labeled protein at room temperature for 16 h, and then the unreacted peptides were removed by gel-filtration. Based on the molecular weight estimation by SDS-polyacrylamide gel electro-
phoresis, one or two molecules of basic peptides and fluorescein per protein were incorporated, respectively.

**Cell Culture**—Mouse macrophage RAW264.7 cells were maintained in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum. Cells were grown on 60-mm dishes and incubated at 37 °C under 5% CO₂ to 70% confluence. A subculture was performed every 3–4 days.

**Peptide Internalization and Visualization**—For each assay, 4 × 10⁴/ml cells were pelleted on an eight-well Lab-Tek II chamber slide (Nalge Nunc) (250 μl/well) and cultured for 16 h. After complete adhesion, the culture medium was exchanged. The cells were incubated at 37 °C for 3 h with the fresh medium (250 μl) containing fluorescein-labeled peptides or proteins. The concentrations of the peptides and proteins were adjusted before addition to the cell based on their respective internalization intensity. Cells were washed three times with PBS, fixed with acetone-methanol (1:1) for 2 min at room temperature, washed three times with PBS again, and then mounted in fluorescent mounting medium containing 15 mM Na₂NO₃ (Dako). The distribution of fluorescein-labeled peptides was analyzed on a Zeiss Axioskop fluorescence microscope equipped with a 100× oil immersion lens.

**Confocal Microscopy**—Cells were grown, incubated with proteins, and fixed basically as described above. Cells were then treated with PBS containing 5 μM propidium iodide (200 μM) at room temperature for 30 min, washed four times with PBS, and mounted in glycerol-PBS (9:1) containing 1% p-phenylenediamine dihydrochloride. Data were obtained using a confocal scanning laser microscope MRC 1024 (Bio-Rad) equipped with a 60× oil immersion lens.

**MTT Assay**—The MTT assay was conducted basically in the same manner as reported previously (2). Cells (1 × 10⁵/well) were cultured in 96-microtiter plates in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum in the presence of peptides (HIV-1 Tat-(48–60): ORKKRRQRRRPQ-amide; R₄₄-Tat: GRRRRRRRRRRPQ-amide; HIV-1 Rev-(34–50): TQQARRRRRREWQR-amide; HIV-1 Rex-(3–16): R₄₄-Rex; and BMV Gag-(7–25) peptide, BMV Gag-(7–25) was fluorescein-labeled for monitoring the internalization of the peptides by fluorescence microscopy. D-Amino acids are denoted in italics. Cysteine residues corresponding to positions 154 and 269 in c-Fos and c-Jun were replaced by serine, respectively.

**RESULTS**

**Uptake of Tat-(48–60) Analogs by the Macrophage Cell**—To obtain insight into the translocation mechanisms of the Tat-(48–60) peptide, Tat-(48–60), its d-amino acid-substituted analog (d-Tat) and arginine-substituted analog (R₄₄-Tat), where residues corresponding to positions 49–57 were replaced with arginine, were synthesized (Fig. 1a). An extra cysteine amide was incorporated into the C terminus of each peptide for the fluorescent labeling. The peptides corresponding to nuclear exportin activity of the HIV-1 Rev-(34–50), FHV coat-(35–49), and nucleoplasmin (14) were also synthesized as references. Treatment of the peptides with 5-maleimido fluorescein diacetate gave the corresponding fluorescein-labeled peptides. Internalization of the peptides was monitored by fluorescence microscopic observation after a 3-h incubation of the peptides with mouse macrophage RAW 264.7 cells at 37 °C. As a result, d-Tat and R₄₄-Tat were internalized into the cell as efficiently as the Tat-(48–60) peptide, and localization into both the cytoplasm and nucleus was observed (Fig. 2). A similar internalization of the d-amino acid analog of Tat was reported by Huq et al. (15) using a linear peptide corresponding to residues 37–72. These results would contradict the idea that a specific receptor may play a crucial role in the translocation of the Tat-(48–60) peptide. On the other hand, the simian virus 40-derived and nucleoplasmin-derived peptides showed a much lower degree of internalization. These NLS-derived peptides are rich in lysine. The above results suggested that arginine residues would play an important role in the translocation.

**Translocation of Various Arginine-rich Nucleic Acid-binding Peptides through the Macrophage Cell Membranes**—Arginine-rich basic segments are used by a variety of RNA-binding proteins to recognize specific RNA structures (16). If arginine-rich basic segments are used by a variety of RNA-binding proteins to recognize specific RNA structures (16). If arginine residues would play an important role in the translocation.

**FIG. 1. Structure of arginine-rich peptides used in this study.** C-terminal cysteine amide (C₄₄) was fluorescein-labeled for monitoring the internalization of the peptides by fluorescence microscopy. D-Amino acids are denoted in italics. Cysteine residues corresponding to positions 154 and 269 in c-Fos and c-Jun were replaced by serine, respectively.

**FIG. 2. Translocation of the arginine-rich Tat-related peptides through the cell membranes.** RAW264.7 cells were treated with fluorescein-labeled peptides derived from HIV-1 Tat-(48–60) (a), R₄₄-Tat (b), d-Tat (c), and nucleoplasmin-NLS (d) (10 μM each) for 3 h. in the sequence plays an important role in the translocation, peptides corresponding to these RNA-binding segments may translocate through the cell membranes. To test this hypothesis, 10 arginine-rich RNA-binding peptides bearing a C-terminal Gly-Cys-amide (Fig. 1b) were similarly prepared, fluorescein-labeled, and applied to the macrophage cells. To our surprise, all the peptides other than the human U2AF-(142–153) peptide translocated through the cell membranes and accumulated in the cytoplasm and nucleus (Fig. 3). As judged from the fluorescent intensity, the efficiency of incorporation into the cells showed a tendency to correspond to the number of arginine residues in the sequence. Internalization activity of the HIV-1 Rev-(34–50), HIV coat-(35–49), HTLV-II Rex-(4–16), and BMV Gag-(7–25) peptides, which have more than seven arginine residues in their sequences, were comparable with that of the Tat-(48–60) peptide. Fluorescence was observed in the cells as early as 5 min after the addition of these peptides (1 μM) to the medium. Less extensive internalization was observed in the case of the λ N-(1–22), d₂₁ N-(12–29), and yeast PRP6-(129–144) peptides that have five arginine residues in their sequences. The fluorescent intensity
in the cells treated with the former peptides (0.1 μM) was judged not to be less than that in those treated with the latter peptides (10 μM). The P22 N-(14–30) and cowpea chlorotic mottle virus Gag-(7–25) peptides that have six arginine residues showed a moderate degree of translocation. HIV-1 Tat-(48–60) is reported to translocate through the cell membranes and accumulate in the nucleus, especially the nucleolus (2). A similar tendency was observed with the above peptides. Not only the RNA-binding peptides but also the DNA-binding peptides corresponding to the basic leucine zipper segments derived from cancer-related proteins, c-Fos and c-Jun, and the yeast transcription factor, GCN4, which were also rich in arginine, were internalized into the cells with almost the same efficiency as that of Tat-(48–60) (Fig. 4).

HIV-1 Tat-(48–60) was reported to induce little toxicity to HeLa cells (2). Using R9-Tat, HIV-1 Rev-(34–50), and FHV coat-(35–49) peptides as representatives of the above arginine-rich peptides, cytotoxicity of the peptides was investigated. Determined by the MTT assay, the above peptides did not show a significant cytotoxicity to the macrophage cells during the treatment with a peptide (10 μM) for 24 h. At 100 μM, cell viability of the cells treated with R9-Tat became 70%, whereas viability of those treated with other peptides as well as HIV-1 Tat-(48–60) was still greater than 95%. These results suggested that many of the arginine-rich peptides can be of low cytotoxicity as reported for the HIV-1 Tat-(48–60) peptide.

Consideration of the Translocation Mechanism of the Arginine-rich Peptides—The above experiments showed that a variety of arginine-rich RNA/DNA-binding peptides were able to translocate through the cell membranes. Little homology in these sequences was observed, except that they all have 5–11 arginine residues. Moreover, the ε-amino acid substituted Rev-(34–50) peptide (1 μM) was internalized as efficiently as the L-peptide in 3 h (data not shown). Circular dichroism (CD) spectra of the HIV-1 Tat-(48–60), R9-Tat, and FHV coat-(35–49) peptides in methanol were suggestive of their not having a significant secondary structure (Fig. 5), whereas the HIV-1 Rev-(34–50) peptide showed a spectrum typical of an α-helical peptide. The U2AF peptide, which was only slightly internalized into the cell, showed a spectrum very similar to that of the FHV coat-(35–49) peptide. These results were suggestive of the absence of even a common secondary structure in the membrane-permeable peptides. When the cells were incubated with a peptide (1 μM) at 4 °C for 30 min, no significant decrease in fluorescent intensity in the cell was observed using the HIV-1 Rev-(34–50), and FHV coat-(35–49) peptides (Fig. 6). These results suggested that typical endocytosis pathways so far established would not play a crucial role in the translocation of these arginine-rich peptides.

We next focused on the question whether the entry of arginine-rich peptides into the cells is one-way or not. The cells were treated with the HIV-1 Rev-(34–50) peptide (1 μM) for 3 h, then the medium was exchanged with a fresh one not containing the peptide. The fluorescence intensity from the cells 1 h later was almost comparable with or only slightly less than that of the cells just before the medium exchange. However, a substantial decrease in the fluorescence intensity was recognized in the cells 6 h later, and complete disappearance of the fluorescence was observed 24 h later. To examine if the above results were due to the leakage of the peptide from the cells, the medium was analyzed by an HPLC equipped with a fluorescence spectrophotometer. No peak was detected at the retention time corresponding to the peptide; however, peaks were observed that eluted at positions identical with those of the peptide treated with trypsin (data not shown). Therefore, we concluded that the decrease in fluorescence intensity of the cells mainly resulted from the degradation of the peptides, and not from the leakage of the intact peptide. The question whether the ingested peptide had a certain effect on the cell growth was also examined. The above HIV-1 Rev-(34–50)-treated cells were harvested 24 h later and counted. The cell number for the peptide-treated cells was comparable with that for the control cells (without peptide treatment). Thus, the peptide-ingesting cells were judged to remain viable to divide with little effect by the peptide. It would be plausible that the peptide evenly distributes in each of the daughter cells upon
cell division, since significant differences in the fluorescence intensity were not observed among the adjoining cells 6 h later. Considering the doubling time of the cell, which was estimated to be about 18 h, a certain amount of cells must have divided within the 6 h. If the peptides would preferentially stay in one of the daughter cells upon cell division, a certain discrepancy in the fluorescence intensity will be observed among the adjacent cells. However, further study will be necessary to adequately address this question.

**Applicability of the Arginine-rich Peptides to the Intracellular Protein Delivery—**

To examine the applicability of the above basic peptides as protein carriers, we prepared basic peptide-protein conjugates. Carbonic anhydrase (29 kDa) was selected as a model protein. Basic peptide-carbonic anhydrase conjugates were prepared using 6-maleimidocaproyloxy)succinimide ester (EMCS) as a cross-linking agent (17) (Fig. 7A). A fluorescein moiety was introduced into the protein using the fluorescein-5(6)-carboxamidocaproic acid N-hydroxysuccinimide ester simultaneously with EMCS. As judged from the SDS-polyacrylamide gel electrophoresis of the conjugates, one to two molecules of the basic peptide and fluorescein moiety were introduced into a molecule of carbonic anhydrase, respectively. Carbonic anhydrase was successfully delivered into the cells with the help of the HIV-1 Rev-(34–50), FHV coat-(35–49), and R9-Tat peptides as efficiently as with the HIV-1 Tat-(48–60) peptide (Fig. 7B). Accumulation of the conjugates in the cytosol and nucleus was also observed by fluorescence microscopy of the cells without fixation (protein concentration: 10 μM) (d). C, confocal microscopic observation of the cells treated with carbonic anhydrase conjugated with the HIV-1 Rev-(34–50) peptide (1 μM) with nucleus staining by propidium iodide (PI) (a). The protein without the carrier peptides (1 μM) did not show a significant accumulation in the nucleus (b).

**FIG. 7.** Delivery of carbonic anhydrase into RAW264.7 cells with the help of arginine-rich basic peptides. A, schematic representation of the conjugates. B, fluorescence microscopy of the cells treated with carbonic anhydrase conjugated with the HIV-1 Rev-(34–50) (a), FHV coat-(35–49) (b), and HIV-1 Tat-(48–60) (c) peptides (1 μM each) for 3 h, respectively. Accumulation of the HIV-1 Rev-(34–50)-carbonic anhydrase conjugate in the cytosol and nucleus was also observed by the fluorescence microscopy of the cells without fixation (protein concentration: 10 μM) (d). C, confocal microscopic observation of the cells treated with carbonic anhydrase conjugated with the HIV-1 Rev-(34–50) peptide (1 μM) with nucleus staining by propidium iodide (PI) (a). The protein without the carrier peptides (1 μM) did not show a significant accumulation in the nucleus (b).

**FIG. 8.** Fluorescence microscopic observation of the cells treated with polyarginine peptides (1 μM) for 3 h (A), and confocal microscopic observation of the cells treated with carbonic anhydrase conjugated with the R₉ or R₁₆ peptides (1 μM) with nucleus staining by propidium iodide (PI) (B).
tein without a carrier peptide was located in a limited part of the cytosol (Fig. 7C). This result suggested that the protein was captured in the endosomes and was not able to be released into the cytosol. Myoglobin (17 kDa) was also introduced into the cell with the help of these carrier peptides (data not shown).

**Effect of the Length of Arginine Chain on the Internalization**—The above data strongly suggested the importance of arginine residues in the internalization. The possible existence of the unique internalization mechanism common in these arginine-rich peptides was also suggested. We then examined the effect of the number of arginine residues in the sequences. For simplification, peptides that are composed of 4–16 residues of arginine were prepared (Fig. 1d). To their C termini, the Gly-Cys-amide segment was also attached for the fluorescein labeling. These results are shown in Fig. 8A. Considerable difference was recognized on the translocation efficiency and intracellular localization among these peptides. R₄ showed extremely low translocation activity. R₅ and R₈ exhibited the maximum internalization and accumulation in the nucleus. What is interesting is that the degree of internalization decreased as the chain length further increased. For the Rₓ₉ internalization of the peptide was not significant. The same kind of difference was recognized in the experiments using the conjugates of carbonic anhydrase with the arginine peptides (Fig. 8B). A similar tendency was observed on the protein delivery using R₄ and Rₓ₉ as the carrier molecules. Based on the confocal laser microscopic observations, the Rₓ-carbonic anhydrase conjugate was efficiently internalized into the macrophage cells and accumulation in the nucleus was observed as was seen in the case of the HIV-1 Rev-(34–50) conjugate. In contrast, the Rₓ₉-conjugate seemed to mainly reside on the cell membranes after a 3-h incubation with the conjugate, but significant accumulation in the nucleus was not observed.

**DISCUSSION**

In this report, we have shown that not only Tat-(48–60) but also various arginine-rich peptides were able to translocate through the mouse macrophage membranes. These peptides include the 0- and arginine-substituted HIV-1 Tat-(48–60) analogs, RNA-binding peptides derived from proteins, such as HIV-1 Rev, HTLV-II Rex, BMV Gag, and HIV coat proteins, and the DNA-binding segments from c-Fos, c-Jun, and the GCN4 proteins. There seems a common or very similar mechanism for the internalization among these peptides. The mechanism is explained neither by adsorptive-mediated endocytosis nor by receptor-mediated endocytosis because the peptides were internalized by the cell at 4 °C, and there seemed little homology both in the primary and secondary structures among these membrane-permeable peptides except that they have several arginine residues in the sequences. These results strongly suggest the possible presence of the common and undefined internalization mechanisms ubiquitously laying among the arginine-rich basic peptides. As one more new finding concerning the features of the internalization, we have shown that the number of arginine residues has a significant influence on the method of internalization and that there seems to be an optimal number of arginine residues for the internalization. There still remain questions why such efficient translocation is possible for the arginine-rich peptides. Possible hydrogen bond formation of arginine with lipid phosphates (18) or interaction with extracellular matrices such as heparan sulfate (19) may be involved in the initial step during the mechanism. However, as was seen in the case of the Rₓ₉ peptide, it is not enough to explain the mechanism only by considering adsorption of peptides on the membranes.

Tat-(48–60) has been reported to carry various proteins into the cells not only into cultured cells but also into the various organs of a living mouse (4). As the arginine-rich peptides examined here seem to have a similar ability as carriers of proteins, further study of the arginine-based peptides may result in finding peptides penetrating to some specific cells by themselves or with the help of other address peptides.

The results obtained here not only shed light on the possible presence of new types of ubiquitous transmembrane mechanisms for the arginine-rich peptides, but also on the development of novel carrier molecules for the intracellular protein delivery.

**Acknowledgments**—We are grateful to Dra. S. Tanaka and Y. Sugimoto (Kyoto University) and Professor H. Harashima (Hokkaido University) for helpful discussions.

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doi: 10.1074/jbc.M007540200 originally published online November 17, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M007540200

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