Translocation of Branched-Chain Arginine Peptides through Cell Membranes: Flexibility in the Spatial Disposition of Positive Charges in Membrane-Permeable Peptides†

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ABSTRACT: A basic peptide derived from HIV-1 Tat has been reported to have the ability to translocate through cell membranes and to bring exogenous proteins into cells. We have demonstrated that these features could be observed among many arginine-rich peptides, and the presence of a ubiquitous internalization mechanism for arginine-rich oligopeptides has been suggested. In this report, we report that these features are also applicable to the peptides having branched-chain structures. Peptides that have arginine residues on four branched chains $(R_n)_4$ [*n* (number of arginine residues) = 0−6] were prepared. Fluorescence microscopic observation revealed that the $(R_2)_4$ peptide exhibited the most efficient translocation. The dependence on the number of arginine residues of the translocation efficiency and cellular localization was also observed for the branched-chain peptides as was seen in the linear peptides. Quite interestingly, efficient translocation was also recognized in the $(RG_3R)_4$ peptide, where three glycine residues intervened between two arginine residues on each chain of $(R_2)_4$. The results strongly suggested that a linear structure was not indispensable for the translocation of arginine-rich peptides and that there could be considerable flexibility in the location of the arginine residue in the molecules.

Basic peptide-mediated protein delivery into living cells has been attracting great attention as a novel technology having potential both for basic research in cellular biology and for therapeutic application. The peptides corresponding to the human immunodeficiency virus type 1 Tat-(48−60) (*1*, *2*) and Antennapedia-(43−58) (*3*, *4*) are among the most well-known peptides for these purposes. By hybridization of these carrier peptides genetically or chemically, efficient intracellular delivery of various oligopeptides and proteins in vitro and in vivo was achieved successfully to modulate cellular events such as cell cycles and apoptosis (*5*, *6*). Even the delivery of a 120 kDa protein, β-galactosidase, into the brain of a mouse in its active form was reported using the Tat peptide as a carrier (*7*). The translocation mechanism also attracts our interest. Although these peptides are highly basic and rich in arginine or lysine residues, they are able to cross the cell membranes to reach the nucleus within a few minutes. In the case of the Tat peptide, it was even internalized efficiently at 4 °C where a typical endocytosis pathway should be considerably suppressed.

We have determined that not only the above peptides but various arginine-rich basic peptides derived from RNA- and DNA-binding peptides, including HIV-1[1] Rev-(34−50), were internalized through the cell membranes and had the ability to bring exogenous proteins into cells (*8*). Quantitative and microscopic studies using HIV-1 Rev-(34−50), HIV-1 Tat-(48−60), and octaarginine (R_8) revealed that these peptides exhibited very similar characteristics in internalization (*9*). We therefore advocated the possibility of the presence of a ubiquitous internalization pathway among the basic peptides. Rothbard et al. also showed the importance of arginine for translocation. Using the heptaarginine peptide, they succeeded in the delivery of cyclosporin A through the skin for inhibiting inflammation (*10*). They also pointed out that the guanidino moiety in arginine plays a crucial role in translocation and suggested the possibility of designing carrier peptides bearing unnatural amino acids (*11*).

On the other hand, branched-chain polymers such as dendrimers have been gaining attention as a novel type of carrier for the delivery of plasmid DNA and drugs into cells (*12*, *13*). Also, branched polypeptides presenting multiple antigen recognition sites have been utilized for vaccination (*14*). If the arginine-rich branched polymers are able to go across the membranes, this will offer great versatility in the design of carrier molecules possessing high efficiency and

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[1] Abbreviations: HIV, human immunodeficiency virus; MAP, multiple-antigen peptide; Fmoc, 9-fluorenylmethyloxycarbonyl; Pbf, N^G-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; SPPS, solid-phase peptide synthesis; DMF, dimethylformamide; DICDI, diisopropylcarbodiimide; HOBt, 1-hydroxybenzotriazole; Trt, *S*-trityl; RP-HPLC, reverse-phase high-performance liquid chromatography; MALDI-TOFMS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; α-MEM, α-minimum essential medium; PBS, phosphate-buffered saline; FHV, flock house virus; CD, circular dichroism; NLS, nuclear localization signal; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; EMCS, *N*-(6-maleimidocaproyloxy)-succinimide ester; PI, propidium iodide.

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cell or organ specificity. Basic peptide-mediated delivery of various molecules, including oligonucleotides, chelating molecules, and even magnetic nanoparticles, has been reported (*15*−*18*). By conjugation of these macromolecules with branched-chain peptides, more sophisticated means of delivery may be realized. Although the translocation of linear peptides has been reported, the necessity of the linear structure for translocation has not been established. Examination regarding this question would have a great implication for understanding the translocation mechanisms of the arginine peptides as well as for the design of novel carrier peptides.

With these hopes in mind, we synthesized several branched-chain arginine-rich peptides. We observed a similar tendency in the translocation efficiency and cellular localization of peptides according to the number of arginine residues. We also showed that these branched peptides also functioned as carriers for intracellular protein delivery. The results suggested that a certain cluster of arginine residues is important for translocation. The difference in the structure of the branched chains resulted in different spectra of cellular localization. The possibility and potential were used in designing a vast array of molecules for intracellular delivery of exogenous molecules into cells.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Fluorescent Labeling. All of the peptides used in this study were chemically synthesized by Fmoc (9-fluorenylmethyloxycarbonyl) solid-phase peptide synthesis on a Rink amide resin as already reported (*19*). Fmoc-Lys(Fmoc) was employed as a building block for branching peptide chains. To facilitate the incorporation of Fmoc-Arg(Pbf)-OH (Pbf is N^G-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl), dimethylformamide (DMF) containing 5% pyridine was used as a solvent for coupling. Four equivalents of Fmoc-amino acid derivatives, diisopropylcarbodiimide (DICDI), and 1-hydroxybenzotriazole (HOBt) per amino function were used for each coupling. Fmoc-Cys(Trt)-OH (Trt is *S*-trityl), Fmoc-Gly-OH, Fmoc-Lys(Fmoc)-OH, and Fmoc-Arg(Pbf)-OH were employed as the Fmoc-amino acid derivatives. Deprotection of the peptide and cleavage from the resin were conducted by treatment with a trifluoroacetic acid/ethanedithiol mixture (95:5) at room temperature for 3 h. R_8 [NH_2-$(Arg)_8$-Gly-Cys-$CONH_2$] and HIV-1 Tat-(48−60) (NH_2-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-Cys-$CONH_2$) were prepared as reported previously (*8*). Fluorescent labeling of the peptides was achieved by treatment with 1.5 equiv of 5-maleimidofluorescein diacetate (Sigma) in a DMF/methanol mixture (1:1) for 3 h followed by reversed-phase HPLC purification. The fidelity of the products was ascertained by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS). Amino acid derivatives and Rink amide resin were purchased from Novabiochem.

Conjugation of Carbonic Anhydrase with Basic Peptides. Conjugation of carbonic anhydrase with branched-chain peptides was similarly carried out as reported previously (*8*). On the basis of the molecular mass estimation by SDS−PAGE, one or two molecules of basic peptides and fluorescein per protein were incorporated.

Cell Culture. Human cervical cancer-derived HeLa cells were maintained in α-minimum essential medium (α-MEM) (Invitrogen) with 10% heat-inactivated calf serum (Invitrogen). Cells were grown on 60 mm dishes and incubated at 37 °C under 5% CO_2 to approximately 70% confluence. A subculture was performed every 3−4 days.

Peptide Internalization and Visualization. For each assay, 1×10^5 cells/mL were pelleted on an eight-well Lab-Tek-II chamber slide (Nalge Nunc) (240 μL per well) and cultured for 48 h. After complete adhesion, the culture medium was exchanged. The cells were incubated at 37 °C with the fresh medium (200 μL) containing fluorescein-labeled peptides or proteins. Cells were washed three times with PBS, fixed with an acetone/methanol mixture (1:1) for 40 s at room temperature, washed twice with PBS again, and then mounted in a glycerol/PBS mixture (9:1) containing 0.1% *p*-phenylenediamine dihydrochloride. The distribution of fluorescein-labeled peptides was analyzed on an IX-70 fluorescence microscope (Olympus) using a 20× lens.

Confocal Microscopy. Cells were grown, incubated with proteins, and fixed in basically the same manner as described above. Cells were then treated with PBS containing 2.5 μM propidium iodide (PI) (200 μL) at room temperature for 10 min, washed four times with PBS, and mounted in a glycerol/PBS mixture (9:1) containing 0.1% *p*-phenylenediamine dihydrochloride. Data were obtained using an LSM 510 confocal scanning laser microscope (Zeiss) equipped with a 40× lens.

RESULTS

Design and Synthesis of Branched-Chain Arginine-Rich Peptides. To investigate whether only linear arginine-rich peptides could penetrate through the cell membranes, we designed the branched peptides shown in Figure 1. Each peptide has a structure having four arginine chains attached to a lysine-based branched chain. At the C-terminus, a cysteine was placed for the fluorescein labeling and a glycine as a spacer between Cys and Lys. Here peptides were named $(R_n)_4$ ($n = 0-6$), where *n* residues of arginine were attached on a four-branched chain. Peptide chains were constructed with the Fmoc solid-phase peptide synthesis method on the Rink amide resin using the DICDI−HOBt coupling system (*19*). Rink amide resin was used to obtain the C-terminal amide structure so that the neutral nature of the C-terminus would facilitate interpretation of the characteristics in the translocation of these basic peptides compared to using peptides bearing a negatively charged carboxylate C-terminus. Fmoc-Lys(Fmoc)-OH was employed for branching the peptide chains, which enabled elongation of the peptide chains on both the α- and ϵ-amino groups. After the introduction of two residues of lysine, arginine chains were constructed simultaneously on the four amino groups derived from the α- and ϵ-positions of the resulting lysine residues on the peptide resin. Usually, DMF has been used as the solvent for coupling of the Fmoc-amino acid with the peptide resin. However, because the inefficiency of introducing Fmoc-Arg(Pbf)-OH onto the branched peptide resin was observed, presumably due to the steric hindrance among peptide chains, DMF containing 5% pyridine was employed as the solvent to improve the incorporation of Fmoc-Arg-(Pbf)-OH. Even with this solvent system, it took 21 h to bring the coupling reaction to completion for introducing the final Fmoc-Arg(Pbf)-OH of $(R_6)_4$. Deprotection and cleavage of the peptides from the resin were conducted by

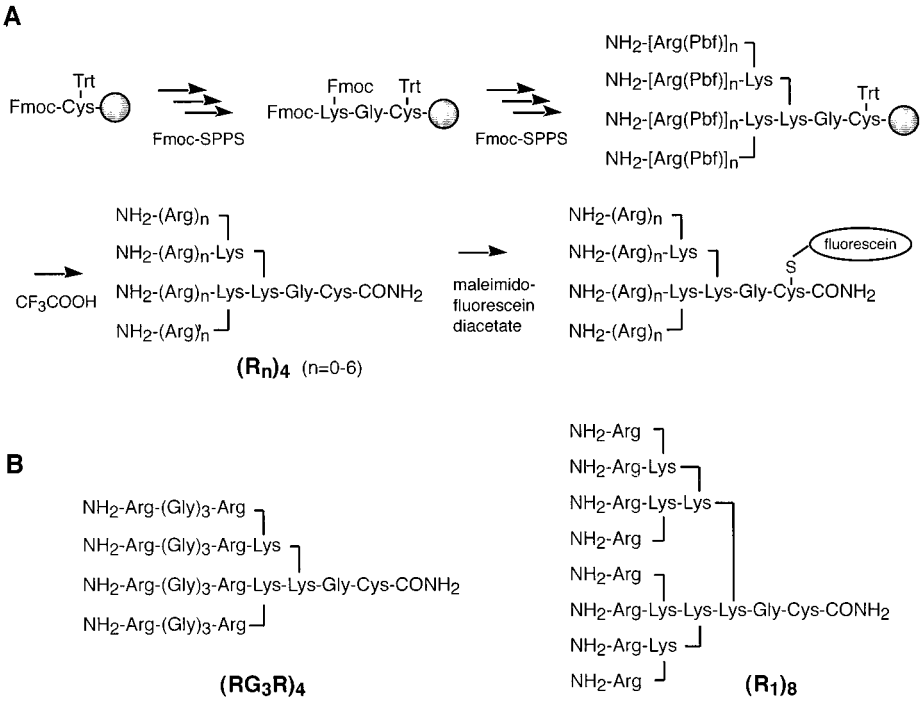

FIGURE 1: Preparation and fluorescein labeling of the branched-chain arginine-rich peptides $(R_n)_4$ (A) and structure of $(RG_3R)_4$ and $(R_1)_8$ (B).

treatment of the peptide resin with a trifluoroacetic acid/ethanedithiol mixture (95:5) at room temperature for 3 h. HPLC purification of the peptide afforded the pure branched-chain peptides (>95% purity determined by analytical HPLC). Fluorescein labeling of the peptides was conducted by treatment with maleimidofluorescein diacetate.

Penetration of Branched Peptide $(R_2)_4$ into HeLa Cells. Various arginine-rich peptides such as HIV-1 Tat-(48−60) as well as oligoarginine have been reported to translocate through the cell membranes (*1*−*11*). We have also reported that not only the Tat peptide but also the translocation of various arginine-rich RNA- and DNA-binding peptides, such as HIV-1 Rev-(34−50) and flock house virus (FHV) coat-(35−49), possessed membrane permeability (*8*). There are almost no sequence homologies among these sequences except that they have approximately eight residues of arginine. The similarity of their secondary structures was not recognized as judged by their CD spectra (*8*). The question of whether the arginine should be linearly arranged for translocation arises. What happens if several arginine residues on the different peptide segments are spherically arranged together to form a basic cluster? In the nuclear localization signal peptides, the sequence from nucleoplasmin (KRPAAT-KKAGQAKKKK) is known to have bipartite basic domains that were separated by 10 intervening spacer amino acids (*20*). It has been assumed that these segments would come close together to form a basic cluster and to be recognized as a nuclear localization signal (NLS) by nuclear transport proteins and delivered into the nucleus. The same concept may be able to be applied to the clusters of arginine residues in functioning as membrane translocation vehicles. Because the octaarginine (R_8) showed the highest translocation activity among the linear oligoarginine peptides (*8*), we examined whether the $(R_2)_4$ peptide was able to translocate through the cell membranes.

HeLa cells were treated with medium containing the fluorescein-labeled $(R_2)_4$ peptide (10 μM) at 37 °C for 3 h. The cells were washed with PBS, fixed, and subjected to fluorescence microscopic observation. As shown in Figure 2A, the peptide $(R_2)_4$ was translocated through the cell membranes very efficiently. Internalization of the peptide was recognized in a substantial portion of the cells. Cellular localization of the peptide appeared to be very similar to that of the linear R_8 peptide (Figure 2A), as well as those of HIV-1 Tat-(48−60) and HIV-1 Rev-(34−50) (not shown), which peptides were reported to show efficient translocation and to function as carriers for intracellular protein delivery. Fluorescence was recognized predominantly both in the cytosol and to a limited extent in the nucleus (presumably the nucleolus). Thus, the linear structure is not necessarily needed for membrane-permeable peptides. Interestingly, there are many other points where $(R_2)_4$ shares the characteristics of translocation with the Tat and R_8 peptides, as described below.

Translocation of Other Branched-Chain Peptides. In our previous study using linear oligoarginine peptides, R_n [NH_2-$(Arg)_n$-Gly-Cys-$CONH_2$ (n = 4−16)], we have shown that not only the translocation efficiency but also the cellular localization of the ingested peptides was determined by the chain length or the number of arginine residues in the sequence (*8*). R_8 was efficiently translocated through the cell membranes and localized in the cytosol and the nucleus. On the other hand, the translocation efficiency of R_4 was quite low compared with that of R_8. Interestingly, as the chain length became greater than eight, the efficiency was again decreased. In the case of the R_{16} peptide, it was observed to reside mainly in the membranes, and localization of the peptide in the nucleus was not significant.

The results attracted our interest regarding whether a similar tendency can be observed in the case of branched peptides. In addition to the above-mentioned $(R_2)_4$ (8 total arginine residues), we have prepared $(R_0)_4$, $(R_1)_4$, $(R_4)_4$, and $(R_6)_4$ (0, 4, 16, and 24 total arginines, respectively). We have also prepared $(RG_3R)_4$ to obtain information regarding

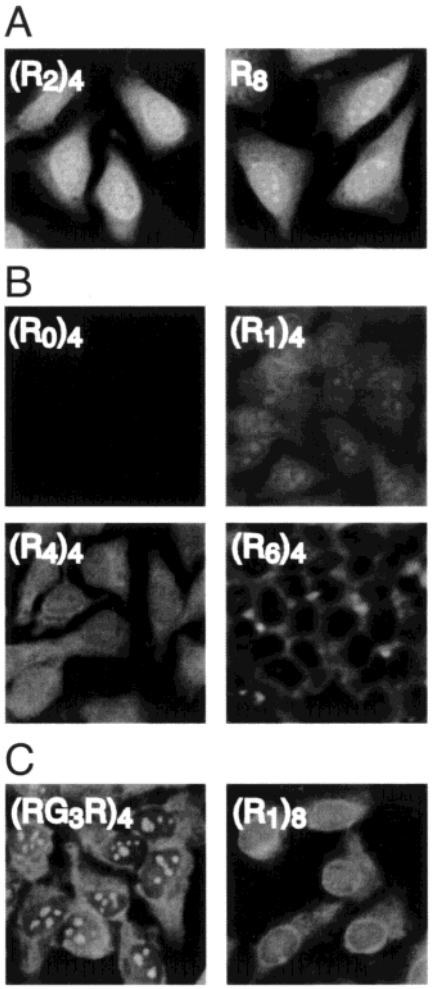

FIGURE 2: Internalization of branched-chain arginine-rich peptides. $(R_2)_4$ was internalized as efficiently as R_8 (A). Dependence on the number of arginine residues of the internalization was observed for the branched peptides as was seen in the linear peptides (B). $(RG_3R)_4$ and $(R_1)_8$ were also internalized efficiently, although the cellular localization of these peptides seemed to be slightly different from that of $(R_2)_4$. The concentration of fluorescein-labeled peptides was 10 μM; treatment was carried out for 3 h.

whether the arginine residues in the branched chain must be located close together. The extra spacer of $(Gly)_3$ in $(RG_3R)_4$ would give a distance of ~10 Å when most extended as roughly calculated from their bond lengths, although the precise conformation on the peptide has not been studied. Other than the above four branched peptides, the $(R_1)_8$ peptide was prepared to confirm if the membrane permeability of the peptides depends on the number of branched chains.

Similar but slightly different tendencies were observed as the means of translocation of $(R_n)_4$ peptides compared with that for the linear arginine peptides. The $(R_0)_4$ peptide exhibited almost no fluorescence in the cell. $(R_1)_4$ showed some internalization, whereas the translocation efficiency of the linear R_4 peptide was quite low. As the number of arginine residues increased, the translocation efficiency also increased. $(R_2)_4$ (8 total arginines) was judged to possess the highest translocation activity and level of nuclear localization. In the case of the $(R_4)_4$ peptide, the internalization of the peptide and accumulation in the nucleus were still observed, whereas in the case of R_{16}, significant accumulation of the peptide in the nucleus was not recognized. The localization

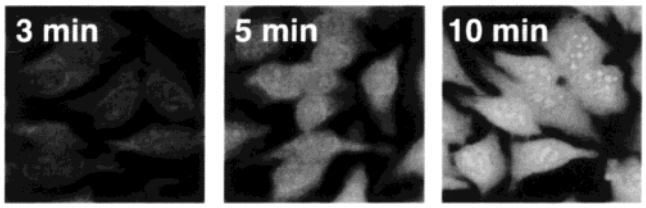

FIGURE 3: Time course of internalization of the $(R_2)_4$ peptide into HeLa cells. The peptide concentration was 10 μM.

of $(R_6)_4$ is rather similar to that of linear R_{16}. The $(R_6)_4$ peptide seemed to reside mainly on the membranes, but little accumulation was observed in the nucleus.

A very interesting result was obtained in the case of $(RG_3R)_4$. The arginine residues in each branched chain were separated via a $(Gly)_3$ linker, a linker which is assumed to have a rather flexible structure and would provide a certain distance between the arginine residues. $(RG_3R)_4$ was translocated as efficiently as $(R_2)_4$, based on fluorescence microscopic observation. The $(R_1)_8$ peptide also showed membrane permeability. Although these peptides showed efficient translocation, their cellular localization, especially that in the nucleus, was subtly different in each case.

Characteristics of Internalization of $(R_2)_4$. In the studies described above, the branched-chain peptides and the linear peptides showed a basically similar manner of translocation, depending on the number of arginine residues in the molecules. To study the manner of internalization more precisely, the experiments were conducted using $(R_2)_4$, which showed one of the most efficient internalizations, as a model.

HIV-1 Tat-(48−60) was reported to show very efficient translocation through the cell membranes to reach the nucleus within 5 min (*2*). Translocation of the $(R_2)_4$ peptide was also very efficient. Figure 3 showed a time-course observation of the internalization of $(R_2)_4$. The fluorescence was recognized within the cells 3 min after the peptide was added to the medium. The extent of internalization increased in an incubation time-dependent manner. After 10 min, fluorescence intensity and cellular localization of the peptide almost comparable to those 3 h later were observed by microscopic observation.

Determined by the MTT assay, the toxicity of the $(R_2)_4$ peptide against the HeLa cells was estimated to be comparable to or slightly higher than that of the R_8 peptide. The viability of the cell, after treatment of the cells with $(R_2)_4$ (10 μM) for 24 h, was 91 ± 2.0%. The viability of R_8 under the same condition was 92 ± 8.5%. At a peptide concentration of 100 μM, viabilities for $(R_2)_4$ and R_8 were 87 ± 1.5 and 96 ± 2.7%, respectively.

With regard to HIV-1 Tat-(48−60), it has been reported that the peptide suffers little inhibition of cellular uptake at 4 °C (*2*). In the case of Antennapedia-(43−58), it has been reported that the level of internalization was slightly reduced but not abolished at 4 °C (*3*). These results suggested that a typical endocytic pathway does not play a crucial role in the internalization of these peptides. A slight decrease in the level of cellular uptake was observed for $(R_2)_4$ by fluorescence microscopic observation after the cells were treated with the peptide (1 μM) at 4 °C for 30 min (Figure 4).

The above-mentioned characteristics observed during translocation of the $(R_2)_4$ peptide were similar to those of the linear arginine-rich peptides, which raised the possibility of them sharing common internalization mechanisms. We

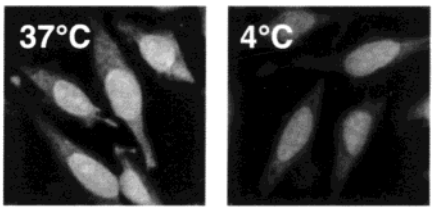

FIGURE 4: Effect of temperature on the internalization of $(R_2)_4$. The peptide concentration was 1 μM; treatment was carried out for 30 min.

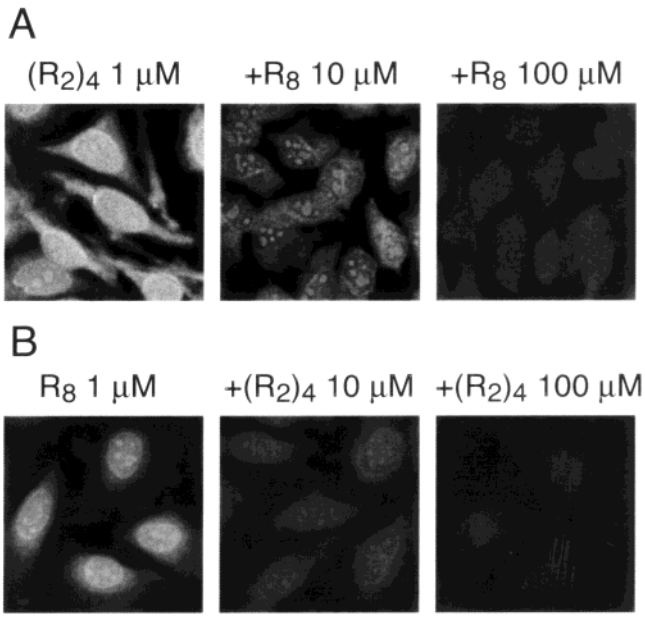

FIGURE 5: Competition of internalization of $(R_2)_4$ in the presence of R_8. Cells were treated with the fluorescein-labeled $(R_2)_4$ (A) or R_8 (B) (1 μM each) in the presence of the other peptides without fluorescein labeling. Treatment was carried out for 3 h.

therefore examined whether the internalization of $(R_2)_4$ can be inhibited in the presence of the R_8 peptide. Cells were treated with $(R_2)_4$ (1 μM) in the presence of HIV-1 Tat-(48−60) or R_8 (100 μM) at 37 °C for 3 h. In the absence of the linear peptides, the $(R_2)_4$ peptide efficiently entered the cell (Figure 5A). However, in the presence of the R_8 peptide, internalization of $(R_2)_4$ was dramatically suppressed. A considerable decrease in the fluorescence intensity of $(R_2)_4$ in the cells was recognized. R_8 also inhibited the uptake of $(R_2)_4$ by the cells (Figure 5B). A similar manner of competition was observed between $(R_2)_4$ and HIV-1 Tat-(48−60) peptides (data not shown).

The $(R_2)_4$ peptide has a carrier activity like that of the Tat-(48−60) and Rev-(34−50) peptides. The $(R_2)_4$ peptide was chemically conjugated with the fluorescein-labeled carbonic anhydrase (CA) (29 kDa) using *N*-(6-maleimidocaproyloxy)succinimide ester (EMCS) as a cross-linking agent as reported previously (*8*). As judged from SDS−PAGE of the conjugates, one to two molecules of the basic peptide and the fluorescein moiety were introduced into a molecule of carbonic anhydrase (data not shown). Cells were treated with the fluorescein-labeled $(R_2)_4$−CA conjugate (1 μM) for 3 h. Confocal microscopic analysis of the conjugate demonstrated a predominant cytoplasmic and nuclear localization and not just attachment to the cellular membranes (Figure 6). The $(RG_3R)_4$ peptide was also shown to have the ability to bring the exogenous protein into the cells. Fluorescence was observed in both the cytosol and nucleus after treatment of the cells with the $(RG_3R)_4$−CA conjugate (1 μM) for 3 h. These results suggested that even a rather wide range of distribution of arginine residues was possible in the carrier. Quite interestingly, the extent of accumulation of the $(RG_3R)_4$−CA conjugate in the nucleolus was assumed to be significantly higher than that of the $(R_2)_4$−CA conjugate based on fluorescence microscopic observation.

DISCUSSION

We have shown in this report that not only linear arginine-rich peptides but also branched-chain peptides were able to

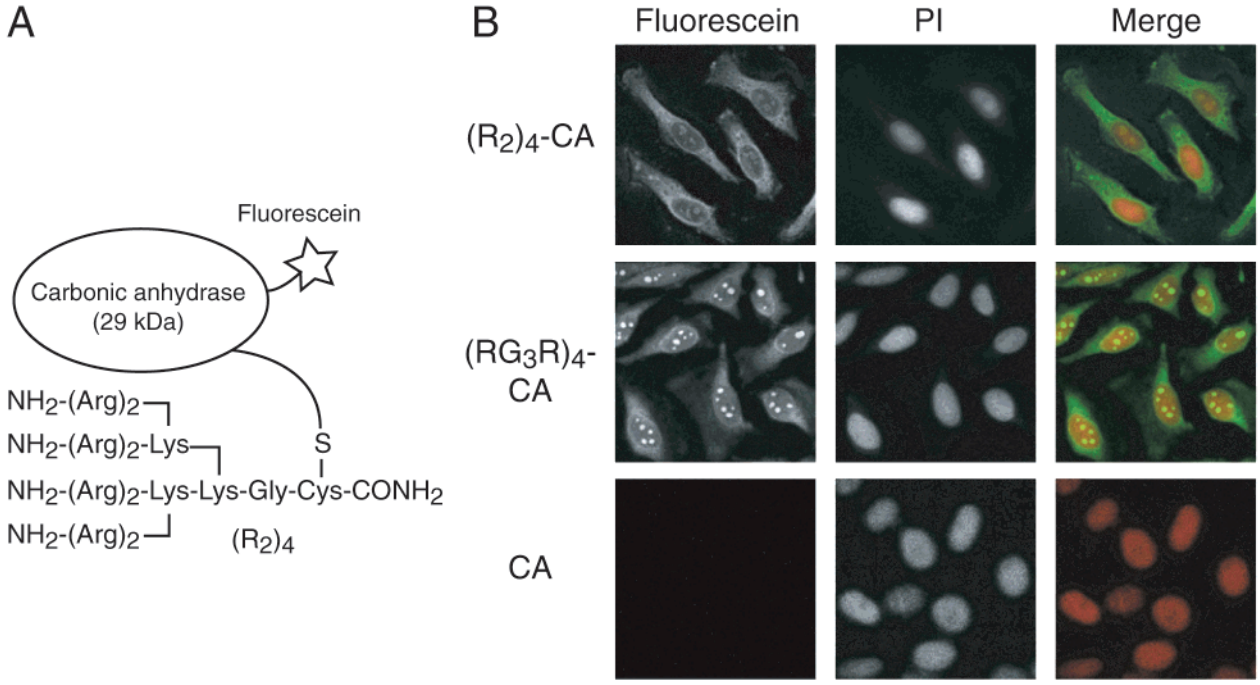

FIGURE 6: Schematic representation of the $(R_2)_4$−CA conjugate (A) and confocal microscopic observation of the cells treated with $(R_2)_4$−CA or $(RG_3R)_4$−CA conjugates (1 μM each) with nucleus staining with propidium iodide (PI) (B).

be translocated through the cell membranes and have the ability to bring exogenous proteins into living cells. Among the peptides that were examined, $(R_2)_4$ (8 total arginine residues) exhibited the most efficient translocation. The larger or smaller the number of arginine residues in the molecules became, the less efficient was the observed internalization. The charge-dependent manner of internalization was basically similar to that observed for the linear R_n peptides (*8*). There are other characteristics similar to those for linear arginine-rich peptides such as HIV-1 Tat-(48−60) and the R_8 peptide; internalization of the $(R_2)_4$ peptide was very fast, reaching the nucleus in a few minutes. The $(R_2)_4$ peptide, like a carrier, has the ability to bring a protein into the cells. Internalization of $(R_2)_4$ was competitively inhibited in the presence of Tat and R_8 peptides. Although a subtle difference was observed in their internalization at 4 °C, these peptides seem to share a significant part of the internalization pathways or the cellular localization sites such as the nucleolus.

Of special interest are the results observed for $(RG_3R)_4$. This peptide does not have a continuous array of arginine residues. The "positive patch" formed by these arginine residues would be larger, and the density of positive charges would become lower than in the case of the linear R_8 and even the branched $(R_2)_4$ peptides. However, the $(RG_3R)_4$ peptide as well as its carbonic anhydrase conjugate was also efficiently delivered into the cells. The result was suggestive of the flexibility in the location of arginine residues in the carrier molecules. The internalization ability observed for $(R_1)_8$ would also support this hypothesis. Thus, for translocation, the arginine residues do not necessarily need to be linearly arranged, and as was seen in the case of $(RG_3R)_4$, continuous arrangement of the arginine residues was not indispensable. These results raise the possibility that a protein bearing a cluster of basic amino acids on its surface might also have the ability to be translocated through the cell membranes. A similar phenomenon was reported by Futami, where the modification of bovine RNase A with ethylenediamine to give positive charges on its surface enhanced the cellular uptake of the protein (*21*).

It is interesting to ask why approximately eight residues of arginine show the highest internalization efficiency. We have recently reported intracellular delivery of plasmid DNA using stearylated oligoarginine peptides (*22*). In this system, the transfection efficiency of the stearyl oligoarginine (stearyl-R_n) coincided with the translocation efficiency of the corresponding oligoarginine peptide (R_n); stearyl-R_8 exhibited a higher transfection efficiency than the stearyl-R_4, -R_{12}, and -R_{16} peptides. The detailed mechanisms of the transfection system have not been clarified at this stage. However, it seems that R_8 provides a key to understanding the above phenomena.

As we discussed, clusters formed by approximately eight residues of arginine play a crucial role in translocation, and there would be a certain flexibility for the position of positive charges in these peptides. Recently, approaches have been introduced to utilize phages and liposomes expressing the Tat peptide for the delivery of DNAs and drugs (*23*, *24*). The concept of the internalization of macromolecules mediated by arginine-rich basic peptides has become more prevalent. The findings described above would provide new insights into the translocation of arginine-rich peptides and the intracellular delivery using these peptides.

REFERENCES

1. Fawell, S., Seery, J., Daikh, Y., Moore, C., Chen, L. L., Pepinsky, B., and Barsoum, J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 664−668.
2. Vivès, E., Brodin, P., and Lebleu, B. (1997) *J. Biol. Chem. 272*, 16010−16017.
3. Derossi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994) *J. Biol. Chem. 269*, 10444−10450.
4. Derossi, D., Chassaing, G., and Prochiantz, A. (1998) *Trends Cell Biol. 8*, 84−87.
5. Nagahara, H., Vocero-Akbani, A. M., Snyder, E. L., Ho, A., Latham, D. G., Lissy, N. A., Becker-Hapak, M., Ezhevsky, S. A., and Dowdy, S. F. (1998) *Nat. Med. 4*, 1449−1452.
6. Vocero-Akbani, A. M., Heyden, N. V., Lissy, N. A., Ratner, L., and Dowdy, S. F. (1999) *Nat. Med. 5*, 29−33.
7. Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. (1999) *Science 285*, 1569−1572.
8. Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., and Sugiura, Y. (2001) *J. Biol. Chem. 276*, 5836−5840.
9. Suzuki, T., Futaki, S., Niwa, M., Tanaka, S., Ueda, K., and Sugiura, Y. (2002) *J. Biol. Chem. 277*, 2437−2443.
10. Rothbard, J. B., Garlington, S., Lin, Q., Kirschberg, T., Kreider, E., McGrane, P. L., Wender, P. A., and Khavari, P. A. (2000) *Nat. Med. 6*, 1253−1257.
11. Wender, P. A., Mitchell, D. J., Pattabiraman, K., Pelkey, E. T., Steinman, L., and Rothbard, J. B. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 13003−13008.
12. Wagner, E. (1999) *Adv. Drug Delivery Rev. 38*, 279−289.
13. Florence, A. T., and Hussain, N. (2001) *Adv. Drug Delivery Rev. 50*, S69−S89.
14. Nardelli, B., and Tam, J. P. (1995) *Pharm. Biotechnol. 6*, 803−819.
15. Astriab-Fisher, A., Sergueev, D. S., Fisher, M., Shaw, B. R., and Juliano, R. L. (2000) *Biochem. Pharmacol. 60*, 83−90.
16. Polyakov, V., Sharma, V., Dahlheimer, J. L., Pica, C. M., Luker, G. D., and Piwnica-Worms, D. (2000) *Bioconjugate Chem. 11*, 762−771.
17. Josephson, L., Tung, C. H., Moore, A., and Weissleder, R. (1999) *Bioconjugate Chem. 10*, 186−191.
18. Lewin, M., Carlesso, N., Tung, C. H., Tang, X. W., Cory, D., Scadden, D. T., and Weissleder, R. (2000) *Nat. Biotechnol. 18*, 410−414.
19. Futaki, S., Ishikawa, T., Niwa, M., Kitagawa, K., and Yagami, T. (1997) *Bioorg. Med. Chem. 5*, 1883−1891.
20. Robbins, J., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1991) *Cell 64*, 615−623.
21. Futami, J., Maeda, T., Kitazoe, M., Nukui, E., Tada, H., Seno, M., Kosaka, M., and Yamada, H. (2001) *Biochemistry 40*, 7518−7524.
22. Futaki, S., Ohashi, W., Suzuki, T., Niwa, M., Tanaka, S., Ueda, K., Harashima, H., and Sugiura, Y. (2001) *Bioconjugate Chem. 12*, 1005−1011.
23. Eguchi, A., Akuta, T., Okuyama, H., Senda, T., Yokoi, H., Inokuchi, H., Fujita, S., Hayakawa, T., Takeda, K., Hasegawa, M., and Nakanishi, M. (2001) *J. Biol. Chem. 276*, 26204−26210.
24. Torchilin, V. P., Rammohan, R., Weissig, V., and Levchenko, T. S. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 8786−8791.

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