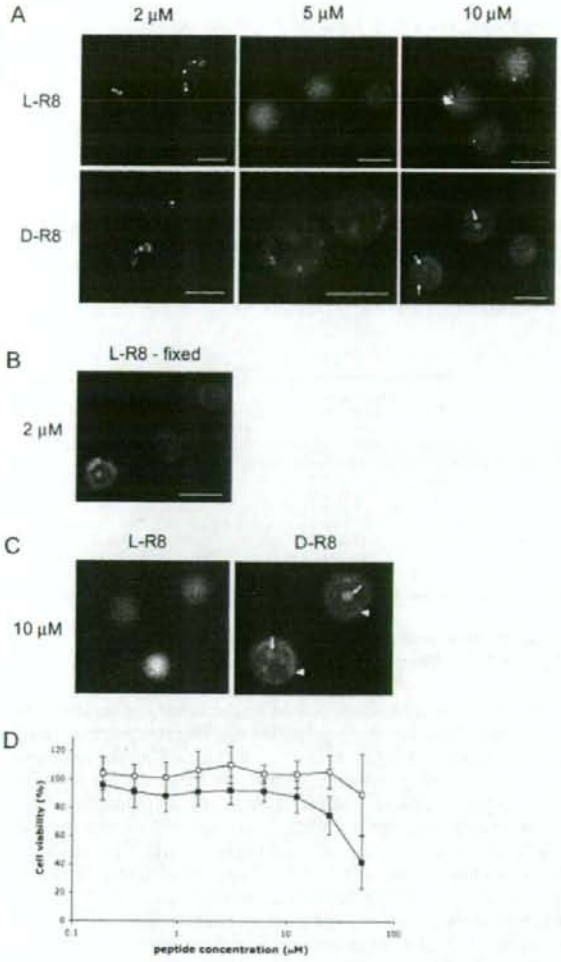

Figure 6 Concentration-dependent cellular distribution and toxicity profiles for R8–Alexa Fluor® 488 peptides

(**A**) KG1a cells were incubated with 2, 5 or 10 μM L- or D-R8–Alexa Fluor® 488 for 1 h at 37 °C before washing and analysis by fluorescence microscopy. Arrows depict labelling of the nucleolus. Scale bars, 10 μm. (**B**) KG1a cells were incubated with 2 μM L-R8–Alexa Fluor® 488 for 1 h at 37 °C. After washing, cells were fixed with paraformaldehyde before analysis by fluorescence microscopy. Scale bar, 10 μm. (**C**) KG1a cells were incubated with 10 μM L- or D-R8–Alexa Fluor® 488 for 10 min at 37 °C before washing and analysis by fluorescence microscopy. Arrows depict labelling of the nucleolus, and arrowheads show faint vesicular labelling. (**D**) Viability of KG1a cells in the presence of increasing concentrations of R8–Alexa Fluor® 488 peptides. KG1a cells were incubated with 0–50 μM L-R8–Alexa Fluor® 488 (□) or D-R8–Alexa Fluor® 488 (■) for 24 h before performing MTT assays. Cell viability is expressed as the percentage of viable cells relative to untreated controls. Results are means ± S.D. for two experiments performed in quadruplicate.

2.5-fold or in plasma membrane cholesterol-depleted cells. These are all conditions that showed microscopical evidence for the presence of this peptide in the cytosol, suggesting this is the critical parameter that then allows for sequestration in the nucleolus. At physiological temperatures, proteolysis is likely to contribute much more to L-R8 degradation compared with D-R8, thus the fraction of this peptide that is able to migrate to the nucleus and nucleolus is likely to be reduced [8,31]. However, we also observed the same differences in localization when the cells were

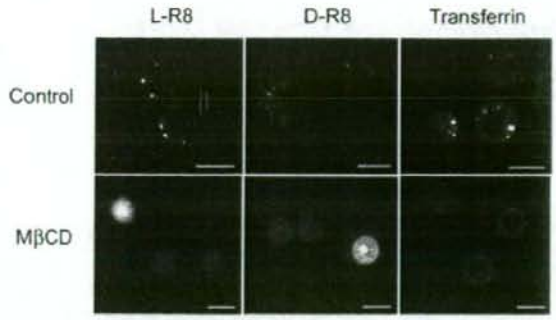

Figure 7 Effects of cholesterol depletion on cellular distribution of R8–Alexa Fluor® 488 peptides and Alexa Fluor® 488–Tf

KG1a cells were pre-incubated in the absence (Control) or presence (MβCD) of 5 mM MβCD for 30 min at 37 °C before washing and incubation with 2 μM L- or D-R8–Alexa Fluor® 488 or 100 nM Alexa Fluor® 488–Tf for 1 h at 37 °C. The cells were then washed and analysed by fluorescence microscopy. Scale bars, 10 μm.

pre-cooled to 4 °C and then incubated with the peptides; here, protease effects should be significantly diminished. L-R8–Alexa Fluor® 488 is clearly able to translocate to the nucleus at low temperatures, and nuclear, as opposed to nucleolar, labelling is often more prominent in L-R8–Alexa Fluor® 488-treated cells (Figure 1). It remains to be seen whether nucleolar sequestration of D-R8 is reducing the extent of the fraction localized to the rest of the nucleus. Nucleolar labelling is a common characteristic of cells that have been incubated with CPPs such as oligoarginine and HIV-Tat and then fixed [7,32,33]. Our results in unfixed KG1a cells do, however, support our previous observations of nucleolar labelling of live HeLa cells incubated with the same D-R8 peptide on ice; the extracellular peptide concentration was, however, appreciably higher at 10 μM [14]. An ability to label the nucleolus was also a feature of other CPPs Flu-β-$(VRR)_4$ and Tat-HA2 [13,34], suggesting that these also have a propensity to bind, most probably, to the RNA that is prominent over DNA in these structures.

Conclusions

We have analysed in detail the effects of temperature, concentration and peptide chirality on the cellular dynamics of R8 peptide in KG1a cells. Our results suggest that L- and D-forms of the peptides share several similarities, such as low-temperature translocation, but also differences with respect to labelling the nucleolus. Enhancing the fraction of the peptide that localizes to the cytosol can be achieved by relatively small increases in peptide concentration or by sequestering plasma membrane cholesterol. These processes seem to be specific for the peptides, as we did not observe any parallel increases in membrane permeability or toxicity. It will now be interesting to investigate whether some of these effects are common to more complex CPPs such as HIV-Tat peptide and penetratin, and also to determine further the capacity of R8 at low temperatures to enhance membrane translocation of associated cargo.

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Acid Wash in Determining Cellular Uptake of Fab/Cell-Permeating Peptide Conjugates

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ABSTRACT:

Successful intracellular delivery of various bioactive molecules has been reported using cell-permeating peptides (CPPs) as delivery vectors. To determine the effects of CPPs on the cellular uptake of immunoglobulin Fab fragment, conjugates of a radio-iodinated Fab fragment with CPPs (CPP-^{125}I-Fab) derived from HIV-1 TAT, HIV-1 REV, and Antennapedia (ANP) were prepared. These vectors are rich in basic amino acids, and their strong adsorption on cell surfaces often results in overestimation of internalized peptides. Cell wash with an acidic buffer (0.2M glycine–0.15M NaCl, pH 3.0) was thus employed in this study to remove cell-surface adsorbed CPP-^{125}I-Fab conjugates. This procedure enabled clearer understanding of the methods of internalization of CPP-^{125}I-Fab conjugates. The kinetics of internalization of REV-^{125}I-Fab conjugate was rapid, and a considerable fraction of REV-^{125}I-Fab was taken up by HeLa cells as early as 5 min after administration. It was also shown that cellular uptake of these conjugates was significantly inhibited in the presence of endocytosis/macropinocytosis inhibitors, in the order REV-^{125}I-Fab > TAT-^{125}I-Fab ≥ ANP-^{125}I-Fab; this order was the same as for effectiveness of intracellular delivery. Simultaneous cell washing with phosphate-buffered saline (PBS) and this acidic buffer effectively separated the internalized conjugates from the cell-surface-adsorbed ones, and considerable differences were observed in these amounts dependent on the employed CPPs. © *2007 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 88: 98–107, 2007.*

Keywords: cell-permeating peptide; antibody; intracellular delivery; acid wash; HIV-1 REV peptide

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INTRODUCTION

Recently, successful delivery of various proteins into living cells has been achieved using cell-permeating peptides (CPPs).[1–5] Among CPPs, a basic peptide segment corresponding to the positions 48–60 of HIV-1 TAT protein, a transcription regulator protein of HIV-1, is one of the most frequently employed for intracellular delivery. This peptide has been used for in vivo delivery of proteins including β-galactosidase and antibodies and

distribution into various organs including liver, lung, kidney, and spleen.[6–11] We have shown that not only TAT but various peptides rich in arginine as well can facilitate translocation.[12–15] Of particular interest is the peptide derived from HIV-1 REV protein, which is known to play a key role in the nuclear export of HIV mRNAs.[16] The peptide corresponding to positions 34–50 of REV protein, the binding site of REV-response element (RRE),[17] has been shown to be highly capable of cell penetration.[11]

We recently reported the distribution of ^{125}I-labeled Fab fragment of immunoglobulin conjugated with REV peptide (REV-^{125}I-Fab) following intravenous administration in rats.[18] On whole-body autoradiography 4 h after administration, REV-^{125}I-Fab produced remarkably higher radioactivity in the adrenal gland, spleen, and liver, than ^{125}I-Fab without the REV segment. We further examined how CPPs affect the patterns of distribution of Fabs similarly administered to rats.[19] The patterns of distribution of ^{125}I-Fab exhibited remarkable variation depending on the CPP employed. In particular, at 4 h, high concentrations of radioactivity were observed in the spleen, adrenal gland, renal medulla, and liver with REV-^{125}I-Fab conjugate, in the liver and spleen with TAT peptide-^{125}I-Fab conjugate, and in the spleen, adrenal gland, and liver with Antennapedia (ANP) peptide (corresponding to positions 43–58 of ANP protein)-^{125}I-Fab conjugate, respectively. We have thus demonstrated that the patterns of distribution of the CPP peptide and immunoglobulin Fab conjugate can be modulated by selection of CPP peptides, and that these CPP-^{125}I-Fab conjugates exhibit different patterns of retention in internal organs. It is of interest, then, to determine the reasons for these differences in distribution. Are there, for example, considerable differences in the method of cellular uptake, although all of these peptides are basic and their physicochemical properties appear not to differ markedly?

Early research suggested the existence of energy-independent pathways of entry of these CPPs and their conjugates with cargo molecules into cells.[20–22] However, more careful and detailed analysis of the methods of internalization of these peptides has revealed that pathways dependent on temperature and ATP (e.g., endocytosis) play crucial roles in their cellular uptake. One of the reasons for the initial misunderstanding of the mechanisms of internalization of CPPs is the strong adsorption of these CPPs to plasma membranes. Insufficient washing of cells treated with CPPs, prior to assessment of efficiency of internalization resulted in inclusion of the peptide on plasma membranes with the peptides actually internalized in cells. When the extent of adsorption was rather high, the observed amount of uptake of CPPs by cells did not significantly decrease even at low temperatures. Care must thus be taken in the analysis or quantification of internalized CPPs.

In this study, to examine whether the differences in efficiency of cellular uptake of CPP-^{125}I-Fab conjugates can explain the differences in their biodistribution, we examined the kinetics and effects of temperature, energy depletion, macropinocytosis inhibitor, and heparin on the cellular uptake of CPP-^{125}I-Fab conjugates. Since antibodies are now considered a promising type of molecular targeting drug, it is also important to examine the methods of cellular uptake of Fabs conjugated to CPPs. Therefore, cellular uptake of three types of chemically conjugated CPP-^{125}I-Fabs, as used in our previous study on biodistribution, were employed; the ^{125}I-labeled immunoglobulin Fab fraction was used as a cargo protein, and REV, TAT, or ANP peptides as CPPs.

In addition, we examined the effects of acid wash in removing cell-surface adsorbed CPP-^{125}I-Fab conjugates on assessment of their cellular uptake. We previously found that arginine-rich CPPs adsorbed on plasma membranes are effectively removed by trypsin treatment of the cell prior to FACS analysis.[23] However, this treatment may not be sufficient for removal of cell-surface-adsorbed CPP-^{125}I-Fab conjugates, since large proteins including Fabs may hinder CPPs from access by trypsin, resulting in incomplete removal of conjugates. Alternatively, treatment of cells with acid buffer has been widely used to remove ligands from various receptors on the cell surface.[24–27] However, to the best of our knowledge, there have been no reports of employment of acid treatment for the assessment of internalization of CPPs. We assessed the usefulness of this treatment for accurate determination of the internalization of CPP-Fab conjugates.

EXPERIMENTAL PROCEDURES

Materials

The native Fab fragment was prepared from a commercially available polyclonal antibody preparation (Venogloblin-IH, Mitsubishi Pharma) using the ImmunoPure Fab Preparation Kit (Pierce) as reported.[18,19] The TAT (GRKKRRQRRRPPQ-C-amide), REV (TRQARRNRRRRWRERQR-GC-amide), and ANP (RQIKIWFQNRRMKWKK-GC-amide) peptides were synthesized using the solid-phase method at the Peptide Institute (Osaka), with extra Cys or Gly-Cys residues attached to the respective CPP segments for chemical conjugation with Fab. The purities of these peptides were 95% or more as determined by HPLC and the structures were confirmed by MALDI-TOFMS.

Preparation of CPP-Fab Conjugates

The Fab fragment was first labeled with ^{125}I by the chloramine-T method.[28,29] The iodinated Fab fragment (^{125}I-Fab) was adjusted to a concentration of 1 mg/mL with a non-labeled Fab fragment solution. Then 120 μg of *N*-(6-maleimidocaproyloxy) succinimide ester (EMCS) was added to ^{125}I-Fab solution (1 mL), and this mixture was gently stirred at room temperature for 2 h. The free EMCS was

removed with a PD-10 column (Amersham) using phosphate-buffered saline (PBS) as eluant. The fractions containing Fab were concentrated by centrifugation using Microprep (Millipore), and adjusted with PBS to a final volume of 1 mL. Two hundred thirty micromol of CPP peptides was added to this reaction mixture, and the mixture was gently stirred at room temperature for 2 h. After the excess portion of peptide was removed using a PD-10 column, the protein fraction was concentrated by centrifugation using Microprep to yield the conjugates of CPP peptides with ^{125}I-Fab (CPP-^{125}I-Fab), the concentration of which was adjusted to 1 mg/mL. The solution of CPP-^{125}I-Fab was then stored below −30°C until use.

The radiochemical purities of CPP-^{125}I-Fab and ^{125}I-Fab were determined by a radio-HPLC method using POROS R2/10 (PerSeptive Biosystems) as an analytical column, and were found to be 97% or more each. In addition, the radiochemical purities of the TCA sediment fraction of CPP-^{125}I-Fab and ^{125}I-Fab were assayed. An appropriate amount of a solution containing 7% bovine serum albumin, 20% TCA, and saline was added to an aliquot of CPP-^{125}I-Fab or ^{125}I-Fab. After this mixture was centrifuged (3000 rpm, 15 min at 4°C) and the supernatant was removed, the sediment was assayed for radioactivity using a gamma counter (Cobra 5005, PerkinElmer). The radiochemical purities of the TCA sediment fractions of CPP-^{125}I-Fab and ^{125}I-Fab were 94% or more each.

Cell Culture

Human cervical cancer-derived HeLa cells were maintained in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS). Cells were grown on 24-well plates and incubated at 37°C under 5% CO_2 to sub-confluence.

Assay for Uptake of CPP-^{125}I-Fab in HeLa Cells

Sub-confluent HeLa cells (24-well plates) were incubated in fresh MEM without FBS (500 μL per well) at 37°C or 0°C (on ice) for 3 h. Five microliter of CPP-^{125}I-Fab (1 mg/mL) was added to each well, and the cells were incubated as shown in the Figure legends. The cells were then washed using either of the following conditions.

i PBS wash: the cells were washed three times with cold PBS (500 μL, 30 sec).

ii Acid wash: the cells were washed twice with cold 0.2*M* glycine buffer containing 0.15*M* NaCl (pH 3.0) (500 μL, 30 sec), followed by cold PBS wash (500 μL, 30 sec).

After washing, 200 μL of trypsin-EDTA solution (Kurabo) was added, and the cells were detached completely from the dishes by incubation at 37°C for 15 min. The radioactivity of cell lysates was then determined using a gamma counter (COBRA Quantum, PerkinElmer).

Cell Viability After Cell Washing

Cell viability after cell wash was assessed by counting cell numbers using Burker–Turk after trypan blue staining.[30] Cell viability was also assessed using the WST-8 kit (Kishida Chemical), which determined the activity of mitochondrial dehydrogenase.[31]

RESULTS AND DISCUSSION

Effects of Acid Wash on Viability of HeLa Cells

The HIV-1 TAT peptide includes positive charges in its sequence, and its strong adsorption to plasma membranes has often resulted in overestimation of internalized peptide.[32] Since the REV and ANP peptides also have positive charges, it is important to remove the particles adsorbed to the membrane to the extent possible for accurate quantification of the peptides taken up by cells.

Glycine buffer (pH 3.0) has often been used for cell washing[33,34] as well as detachment of antibody from antigen.[35,36] The adsorption of the CPPs to plasma membranes is mainly attributed to their interaction with negatively-charged proteoglycans on plasma membranes. Acid wash should be effective in disrupting the interaction between these molecules. Although acid wash has already been employed for cell washing, it might damage cell membranes. The integrity of the plasma membranes was thus examined prior to study of cellular uptake of CPP-Fab conjugates.

Acid wash was performed on HeLa cells maintained in MEM, using 0.2*M* glycine buffer containing 0.15*M* NaCl (pH 3.0). Since the possibility could not be ruled out that CPP-Fab conjugates damage cells, acid wash was performed in this experiment in the absence of CPP-Fab conjugates to assess the effects of acid wash itself. After cell washes (4×) with this acidic buffer, no significant morphological change was noted on microscopic observation compared with cells washed with PBS (Figure 1A). When the cytotoxicity of this wash was assessed using trypan blue staining,[30] little differences in live cell numbers were noted from those after PBS washing (Figure 1B). The lack of significant toxicity of this acid wash was also confirmed by analysis of mitochondrial dehydrogenase activity (Figure 1C).

Kinetics of Cellular Uptake of REV-^{125}I-Fab and Glycine-Buffer Wash of Cells

After confirming that acid wash does not markedly damage cells, we used it for assessment cellular uptake of REV-Fab conjugate using ^{125}I-labeled Fab (REV-^{125}I-Fab). HeLa cells were treated with REV-^{125}I-Fab for 5–60 min and then washed with PBS. After trypsinization, the radioactivity from the cells was analyzed (open circles in Figure 2). Rapid increase in cell-associated radioactivity was observed in the first 30 min, with slower increase observed thereafter. About 13% of total radioactivity had been obtained from the cells under the above conditions at 60 min.

When the cells were washed with the glycine buffer (pH 3.0) after the same treatment with REV-^{125}I-Fab, an ~25%

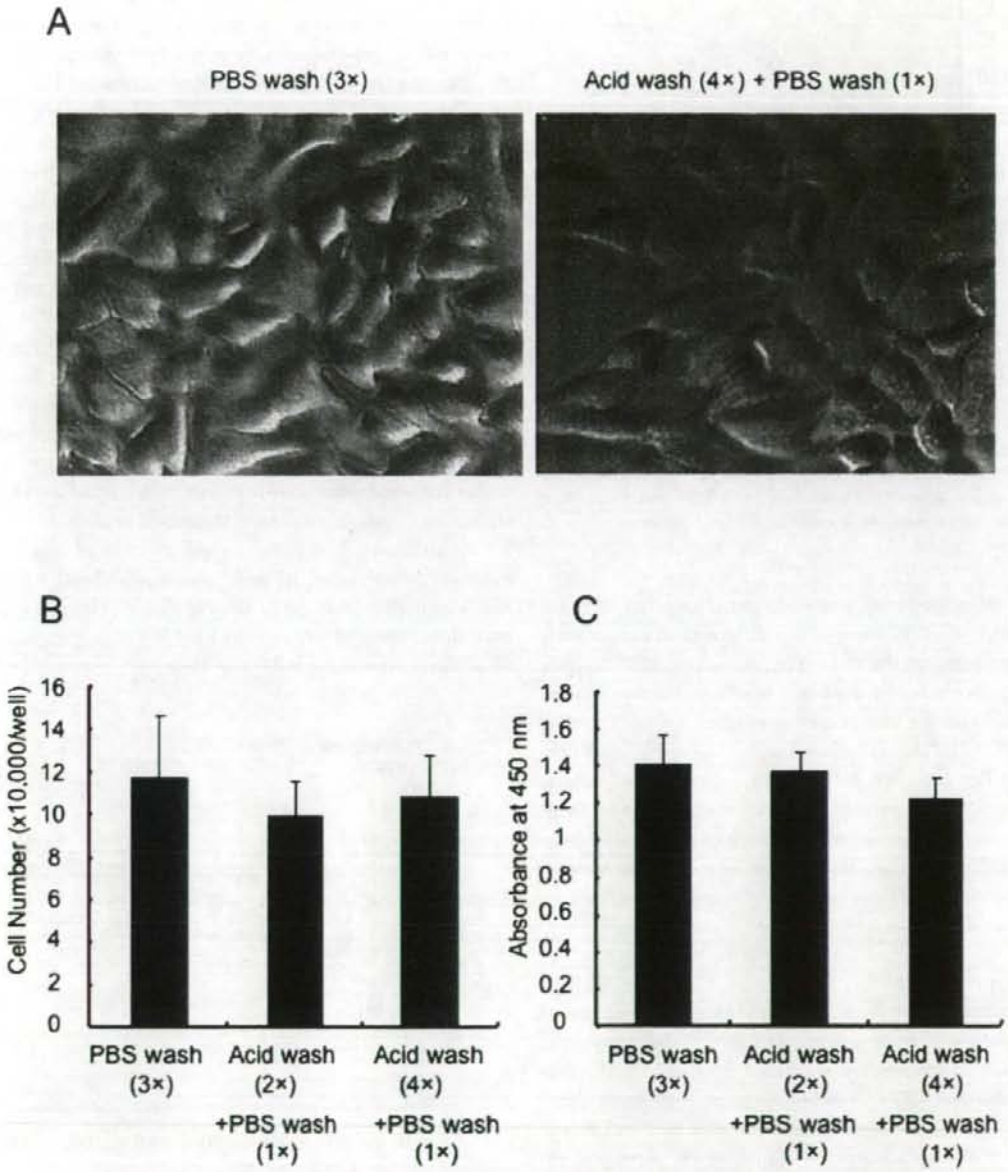

FIGURE 1 Little cytotoxic effect of acid wash of HeLa cells. A: Phase-contrast microscopic observation (×200) of HeLa cells washed with PBS (three times) (left) and acid wash using 0.2*M* glycine buffer containing 0.15*M* NaCl (pH 3.0) (4 times) followed by PBS wash (right). B: Effect of acid wash on cell viability examined by trypan blue staining. Vertical axis corresponds to the numbers of live cells per well. Error bars represent the mean ± standard deviation (SD) for 3 samples. C: Cytotoxicity of acid wash (mitochondrial dehydrogenase assay). Vertical axis represents the enzyme activity of live cells. Error bars represent the mean ± SD for 3 samples.

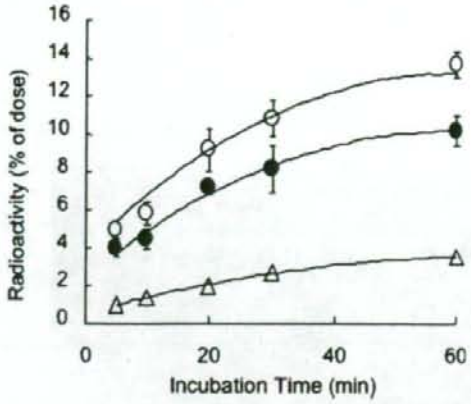

FIGURE 2 Kinetics of cellular uptake of REV-^{125}I-Fab and effects of acid wash. HeLa cells were incubated with REV-^{125}I-Fab (1 mg/mL) at 37°C for the time indicated, washed with PBS (open circles) or 0.2*M* glycine buffer containing 0.15*M* NaCl (pH 3.0) (closed circles), and then trypsinized. Open triangles indicate the difference in radioactivity between cells washed with PBS and the glycine buffer. Error bars represent the mean ± SD for 3 samples.

decrease in radioactivity was observed, compared with the cells washed with PBS. Most of this difference in radioactivity can be attributed to the cell-surface-adsorbed REV-^{125}I-Fab, since the glycine buffer does not markedly damage plasma membranes and the internalized conjugates are not removed by the cell wash. These findings suggest the usefulness of the glycine buffer (pH 3.0) for accurate estimation of cellular uptake of CPP-Fab conjugates. Since simple trypsin treatment was sufficient for removal of surface-adsorbed oligoarginine peptide,[23] additional care must be paid to the removal of CPPs when they are conjugated with cargo molecules.

Chase Experiments

After confirming that the acid wash with glycine buffer (pH 3.0) is effective in removing excess CPP-^{125}I-Fab conjugates on plasma membranes, we examined how quickly the cell-surface adsorbed CPP-^{125}I-Fab conjugates were internalized into cells. HeLa cells were incubated with REV-^{125}I-Fab at 37°C for 5 min to adsorb the conjugate on plasma membranes. Subsequently, the culture medium was replaced with fresh MEM not containing REV-^{125}I-Fab. In this case, no extensive cell wash was performed, in order not to affect cell-adsorbed conjugates. The cells were further incubated at 37°C and then washed with PBS or glycine buffer. The radioactivity of cell lysate after incubation for 5 (when the medium was replaced), 10, 30, and 60 min followed by cell wash with one of these buffers was then examined (Figure 3).

The cell-associated radioactivity obtained after the PBS wash was nearly constant over 60 min. This can be assumed to be total cell-associated radioactivity. On the other hand, more than a two-fold increase in residual radioactivity from the cells washed with glycine buffer was observed in 60 min compared with 5 min; this reflects the radioactivity of the internalized conjugates.

Assuming that the radioactivity from the PBS-washed cells reflects total cell-associated REV-^{125}I-Fab and that from acid-washed cells the internalized portion of it, these findings indicate that almost 40% of the total cell-associated REV-^{125}I-Fab was internalized as early as within 5 min after administration of the conjugate to cells. On the other hand, the residual radioactivity from the acid-washed cells after 60 min of administration was almost identical to that from PBS-washed cells, suggesting that almost all of the cell-adsorbed REV-^{125}I-Fab was internalized within this period. This early internalization of REV-^{125}I-Fab was also observed under the conditions shown in Figure 2. In the experiment shown in Figure 2, cells were incubated with REV-^{125}I-Fab for the indicated time without medium change, whereas the medium was changed to fresh medium without REV-^{125}I-Fab 5 min after addition of the conjugate (Figure 3). However, the amount of internalized REV-^{125}I-Fab at 60 min (the result for acid-washed cells) was about 2.5 times as large as

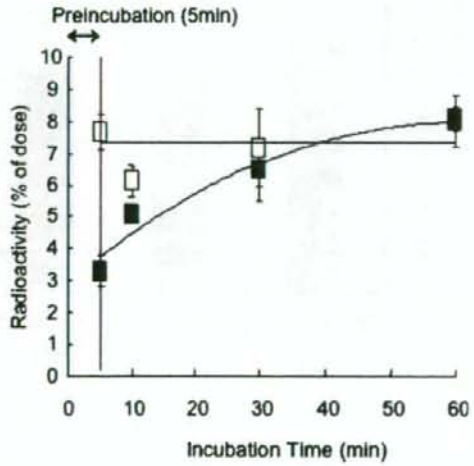

FIGURE 3 Time course of cell-associated radioactivity after 5 min treatment of REV-^{125}I-Fab. HeLa cells were incubated with REV-^{125}I-Fab at 37°C for 5 min. Medium was replaced with fresh medium without REV-^{125}I-Fab, and cells were incubated up to 60 min. After washing of the cells with PBS (open squares) or 0.2*M* glycine buffer containing 0.15*M* NaCl (pH 3.0) (closed squares) at the indicated times, cell-associated radioactivity was examined. Error bars represent the mean ± SD for 3 samples.

that at 5 min in both cases, suggesting that the majority of REV-^{125}I-Fab accumulated on cell surfaces as early as 5 min after addition of REV-^{125}I-Fab to the medium.

Effectiveness of Glycine-Buffer Wash for Analysis of Methods of Internalization of Fab Conjugated With Peptide Vectors

We previously reported that conjugation of Fab with REV, TAT, and ANP peptides leads to efficient internalization of the conjugates in cells.[19] Intravenous injection of these conjugates in rats yielded vector-dependent biodistribution of the conjugates. Since the details of the mechanisms of uptake of these conjugates have not yet been determined, we examined them in this study using the acid wash method.

First, to determine whether uptake of these conjugates is energy-driven, the uptake of CPP-^{125}I-Fab in HeLa cells was examined at 0°C as well as 37°C (Figure 4). HeLa cells were treated with these conjugates for 30 min at the respective temperatures, washed, and cell-associated radioactivities were determined.

At 37°C, the efficiency of internalization was REV-^{125}I-Fab > TAT-^{125}I-Fab ≥ ANP-^{125}I-Fab, as already reported.[19] The amounts of cell-associated radioactivity with the respective conjugates after glycine-buffer (pH 3.0) wash were almost half those after PBS wash, suggesting that much of the conjugates remained on the cell surface without being internalized at 30 min. High levels of radioactivity were also observed for cells even with treatment at 0°C followed by PBS wash. On the other hand, the radioactivity from cells treated at 0°C with glycine-buffer (pH 3.0) wash was marginal and markedly lower than that after PBS wash. This suggests that the strong cell-surface adsorption of CPP-^{125}I-Fab conjugates can result in incorrect assessment of the methods of internalization of CPP-protein conjugates, and that acid wash using glycine buffer (pH 3.0) is an efficient way of removing these surface-adsorbed proteins. When the cells were washed with glycine buffer (pH 3.0) after treatment with CPP-^{125}I-Fab conjugates, the radioactivities from 0°C-treated cells were 29, 19, and 34% of those from the 37°C-treated REV-, TAT-, and ANP-^{125}I-Fab conjugates, respectively. This suggested that an energy-dependent pathway is involved in the cellular uptake of these conjugates.

Effects of NaN_3

After confirming the usefulness of the acid wash procedure for estimation of internalized CPP-conjugates, we applied this method to assess the effects of NaN_3 on the cellular uptake of CPP-^{125}I-Fabs. NaN_3 is a well-known inhibitor of adenosine triphosphate (ATP), and endocytic uptake of the

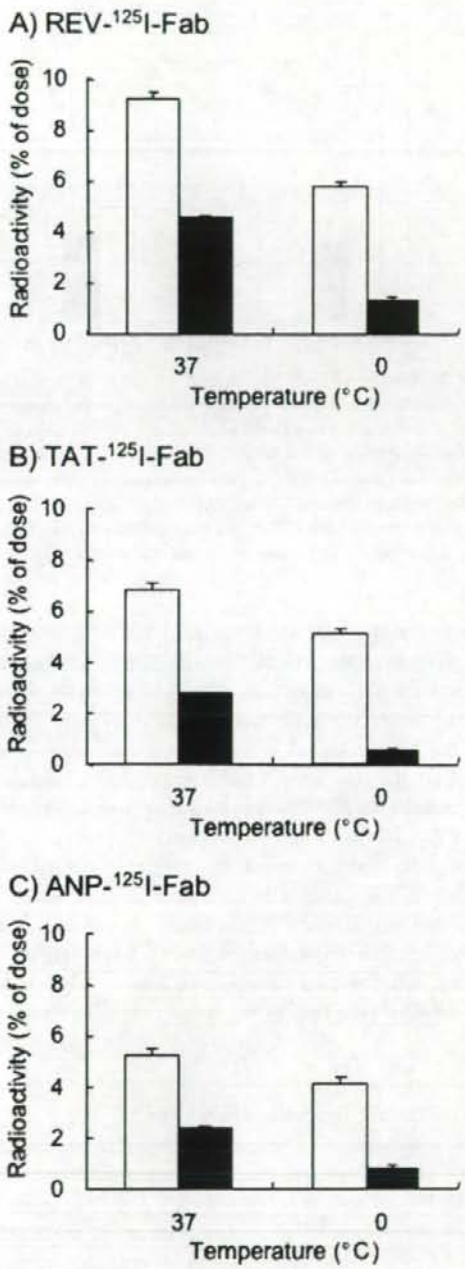

FIGURE 4 Effects of temperature on uptake of CPP-^{125}I-Fab conjugates by HeLa cells. HeLa cells were incubated in MEM without FBS at 37°C or 0°C for 3 h prior to addition of CPP-^{125}I-Fab conjugates. After incubation of the cells at the respective temperatures for 30 min followed by cell wash with PBS (open bars) and 0.2*M* glycine buffer containing 0.15*M* NaCl (pH 3.0) (closed bars), cell-associated radioactivity was examined. Error bars represent the mean ± SD for 3 samples.

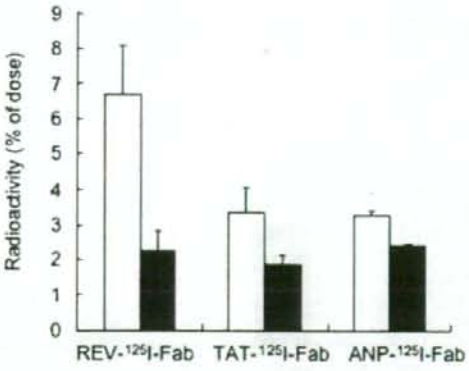

FIGURE 5 Effects of NaN_3 on uptake of CPP-^{125}I-Fabs. HeLa cells were incubated in MEM containing 1% NaN_3 in the absence of FBS at 37°C for 30 min prior to the addition of CPP-^{125}I-Fab conjugates. After incubation of the cells at 37°C for 30 min, cell-associated radioactivity due to CPP-^{125}I-Fabs was examined using the glycine-buffer wash procedure (closed bars). As a control, the cells were similarly treated with CPP-^{125}I-Fab conjugates in the absence of NaN_3 (open bars). Error bars represent the mean ± SD for 3 samples.

conjugates should thus be suppressed by this treatment. Radioactivity from the cells treated with CPP-^{125}I-Fabs in the presence of 1% NaN_3 at 37°C for 30 min was therefore examined using the glycine-buffer wash procedure (Figure 5).

Significant decrease in cell-associated radioactivity was observed for the cells treated with the respective conjugates in the presence of NaN_3, suggesting that internalization of these CPP-^{125}I-Fabs is ATP-dependent. The most notable effect of NaN_3 was observed for the cells treated with REV-^{125}I-Fab, whereas its effect on the uptake of ANP-^{125}I-Fab was not pronounced. Temperature also exhibited the least effect on cellular uptake of ANP-^{125}I-Fab (Figure 4), suggesting that the internalization of ANP-^{125}I-Fab is less energy-sensitive than that of the other two ^{125}I-Fab conjugates.

Effects of Macropinocytosis Inhibitor

Recently, involvement of macropinocytosis in the cellular uptake of octaarginine and the TAT peptides has been demonstrated by us and another group.[23,37–39] We therefore examined the possibility of its involvement in the cellular uptake of CPP-^{125}I-Fab conjugates. 5-(*N*-ethyl-*N*-isopropyl) amirolide (EIPA) is a typical inhibitor of macropinocytosis. HeLa cells were treated with 100 μM EIPA at 37°C for 30 min. After the respective CPP-^{125}I-Fab conjugates were added to the cells, cells were further incubated with the conjugates in the presence of 100 μM EIPA for 30 min, and radioactivity was measured after glycine-buffer wash (Figure 6).

Significant inhibition by EIPA of cellular uptake of CPP-^{125}I-Fab conjugates was observed, suggesting the involvement of macropinocytosis as well in Fab uptake using CPPs. Interestingly, the effect of EIPA on uptake of ANP-^{125}I-Fab was also smaller than that for the other two conjugates, as observed for the effects of temperature and NaN_3 on cellular uptake. We previously reported that the effect of EIPA on cellular uptake of ANP peptide itself is smaller than that for octaarginine.[37] Translocation of ANP peptide into artificial membranes has also been reported.[40] Therefore, although endocytosis plays a certain role in the cellular uptake of ANP conjugate, this may not be the sole pathway of its internalization.

Co-Incubation With Heparin

Membrane-associated proteoglycans have been reported to be very important to the internalization of REV,[13] TAT,[41–43] and ANP[44] peptides. Heparin should competitively inhibit the adsorption of CPP-^{125}I-Fabs onto the membrane-associated proteoglycans on cell surfaces. We have reported that conjugation of ^{125}I-Fabs with CPPs yields different biodistributions and retention patterns in rats via selection of CPPs including REV, TAT, and ANP.[18,19] It can be assumed that differences in biodistribution and retention are due to differences in the affinity of CPPs for membrane-associated proteoglycans on various organs. Although there are reports on inhibition of CPP uptake by heparin, most employed rather high concentrations of heparin for clear exhibition of inhibitory effects. Detailed study of the relationship between the

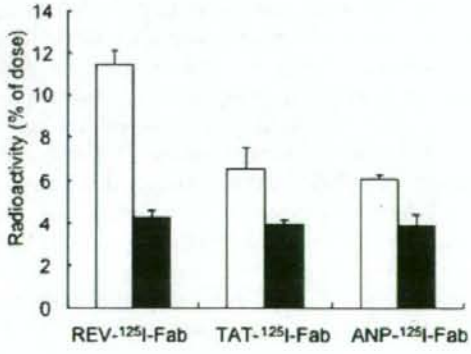

FIGURE 6 Effects of macropinocytosis inhibitor (EIPA) on cellular uptake of CPP-^{125}I-Fabs. HeLa cells were treated with 100 μM EIPA in MEM without FBS at 37°C for 30 min prior to addition of CPP-^{125}I-Fab conjugates. After incubation of the cells at 37°C for 30 min, cell-associated radioactivity was examined using the glycine-buffer wash procedure (closed bar). As a control, the cells were similarly treated with the CPP-^{125}I-Fab conjugates in the absence of EIPA (open bar). Error bars represent the mean ± SD for 3 samples.

structures of CPPs and their affinity for proteoglycans would yield findings valuable for selective delivery using CPPs. We therefore examined the cellular uptake of CPP-^{125}I-Fab conjugates within 30 min in the presence of varying amounts of heparin in relatively low concentrations (0–0.5 U/mL). Here, the results obtained after PBS wash and glycine-buffer wash are compared.

Uptake of CPP-^{125}I-Fabs was greatly suppressed in the presence of heparin. When the cells were treated with REV-^{125}I-Fab conjugate in the presence of 0.1 or 0.5 U/mL heparin, for example, the cell-associated radioactivity after PBS wash was almost 60 and 15% of that in the absence of heparin, respectively. In the absence of heparin, acid wash yielded 55% of the radioactivity after PBS wash, suggesting that a considerable portion of cell-associated radioactivity (ca 45%) was from cell-surface adsorbed REV-^{125}I-Fab conjugate. In the presence of 0.1 U/mL heparin, radioactivity of the acid-washed cells was about 80% of that of the PBS-washed cells; in the presence of 0.5 U/mL heparin, the radioactivities in the two cases were almost identical. Assuming the differences in radioactivity between PBS-washed cells and acid-washed cells represent the amount of cell-surface adsorbed REV-^{125}I-Fab conjugate, equilibrium between the membrane-associated proteoglycans and the externally added heparin was effectively shifted to the heparin side by its addition, and this eventually reduced the amount of cell surface adsorbed conjugate over that taken up by the cells. In the presence of 0.5 U/mL heparin, decrease in radioactivity of the PBS-washed cells was 85% of that in its absence. However, the decrease in that of the acid-washed cells was 70%. These results suggested the possibility of overestimation of the inhibitory effect on uptake of heparin when removal of cell-surface adsorbed CPP conjugates was ineffective. They also indicate the usefulness of the acid wash procedure for accurate estimation of cellular uptake of CPP-conjugates.

In the case of TAT-^{125}I-Fab conjugate, a marked decrease in cellular uptake was also observed in the presence of heparin. The radioactivity of PBS-washed cells treated with TAT-^{125}I-Fab in the presence of heparin (0.1–0.5 U/mL) was almost twice that of the acid-washed cells. Notably, however, the difference in radioactivity between the PBS-washed cells and acid-washed cells was almost the same for the TAT and REV conjugates. This suggests that the amount of surface adsorption of these conjugates is almost the same, whereas the REV peptide has higher capacity for delivery of Fab into cells than TAT peptide, resulting in stronger shift of the REV-^{125}I-Fab conjugate than of TAT-^{125}I-Fab conjugate into cells from medium. In marked contrast to the case of these peptides, the cell-associated radioactivities after PBS and acid wash of ANP-^{125}I-Fab conjugate were almost identical in the

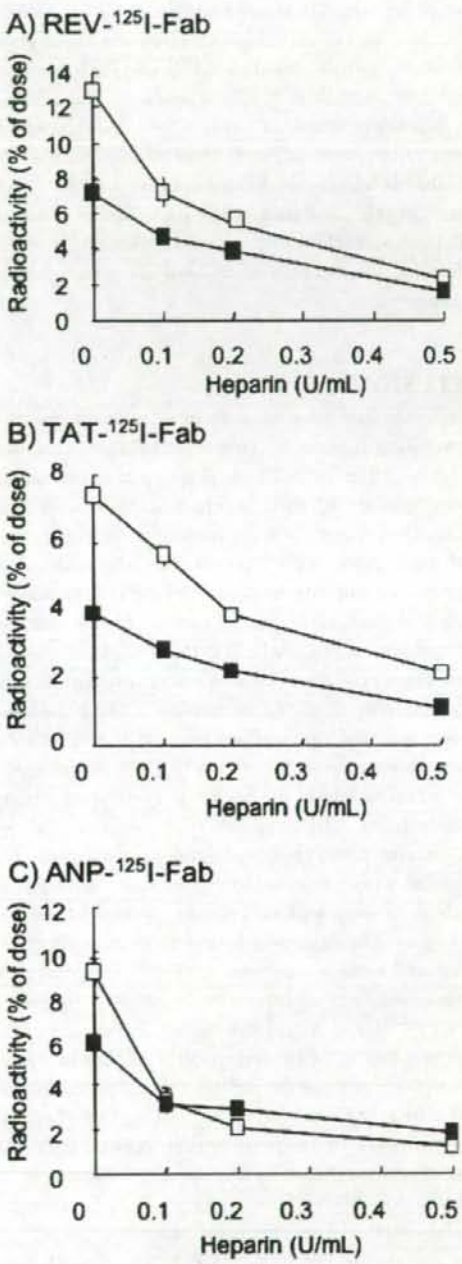

FIGURE 7 Effects of heparin on uptake of CPP-^{125}I-Fab in HeLa cells. HeLa cells were pretreated with heparin in MEM without FBS at 37°C for 5 min. After the addition of CPP-^{125}I-Fabs, cells were incubated at 37°C for another 30 min. Radioactivity due to CPP-^{125}I-Fab was measured after cell wash with PBS (open squares) or 0.2*M* glycine buffer containing 0.15*M* NaCl (pH 3.0) (closed squares). Error bars represent the mean ± SD for 3 samples.

presence of heparin, suggesting that the affinity of ANP-^{125}I-Fab conjugate for cell surface proteoglycans is much smaller than those of its corresponding REV- and TAT-conjugates. Although further study is of course necessary, these differences in efficiency of translocation of CPP-^{125}I-Fab conjugates and their affinity for cell-surface proteoglycans may help explain the differences in biodistribution of CPP-^{125}I-Fab conjugates in rat,[19] and suggest the usefulness of simultaneous PBS wash and acid wash for analysis of methods of internalization and interaction with cell-surface proteoglycans of CPP conjugates.

CONCLUSION

Recently, antibodies have come to be considered one of the most promising classes of pharmaceutical agents useful for molecular targeting. Intracellular delivery of antibodies may open new avenues of their therapeutic application. Since antibodies are proteins of high molecular weight, development of methodologies for efficient intracellular delivery of them is necessary for this purpose. We have recently reported the successful delivery of Fab to various organs in rats by conjugation with CPPs, including the TAT, REV and ANP peptides. These conjugates yielded rather different biodistributions depending on the CPPs employed. Since few studies have been reported the mechanisms of internalization of CPP-Fab conjugates and the effects of CPPs on internalization, we examined here the methods of internalization of CPP conjugates of ^{125}I-labeled Fab.

For assessment of cell-associated radioactivity from CPP-^{125}I-Fab conjugates, we employed two washing procedures, the PBS wash and acid (0.2*M* glycine–0.15*M* NaCl, pH 3.0) wash. The latter was found to be very effective in removing cell-surface adsorbed CPP-^{125}I-Fab conjugates. Using these wash procedures simultaneously, internalization of CPP-^{125}I-Fab conjugates within 5 min after administration was found to be almost 50% of that in 60 min. Cellular uptake of these conjugates was inhibited by treatment of cells at 0°C and NaN_3, suggesting that energy-dependent pathways are involved in their cellular uptake. Inhibition of their cellular uptake by EIPA treatment also suggests the possibility of involvement of macropinocytosis in cellular uptake. The effects of these treatments were stronger in the order REV-^{125}I-Fab > TAT-^{125}I-Fab $\geq$ ANP-^{125}I-Fab (Figure 7). Since this order coincides with the efficiency of their cellular uptake at 37°C, endocytosis or macropinocytosis may increase the uptake of REV conjugates more effectively than that of other conjugates. We also demonstrated differences between these conjugates in their affinity for cell surface proteoglycans in the presence of heparin. Although further studies are necessary, we believe that the findings obtained here provide information valuable for understanding the mechanisms of uptake of CPP-antibody conjugates as well as the design of antibody delivery systems using CPPs.

The usefulness of the acid wash procedure employed in this study deserves mention. Trypsin treatment is an effective means of removing cell-surface adsorbed small peptides bearing CPPs. However, this procedure may not sufficiently effective in removing proteins of higher including antibodies, as reported here, due to insufficient enzymatic digestion of the target proteins on cell surface or difficulty in determination of optimal conditions for enzymatic digestion. Trypsin treatment may be similarly ineffective in removing non-peptide cargoes such as synthetic polymers and liposomes. However, acid wash should be useful for this purpose. The simultaneous employment of both PBS and acid wash procedures should be a simple way of assessing internalized CPP conjugates versus cell-surface-adsorbed ones, as exemplified in this study.

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REVIEW

Ligand-Induced Extramembrane Conformation Switch Controlling Alamethicin Assembly and the Channel Current

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In this review, we describe our approach to creating artificial receptor-channel proteins or sensor systems, using an extramembrane segment conformationally switchable by external stimuli. Alamethicin is known to self-assemble in membranes to form ion channels with various open states. Employment of an α-helical leucine-zipper segment resulted in the effective modulation of the association states of alamethicin to produce a single predominant channel-open state. A decrease in the helical content of the extramembrane segments was found to induce a channel-current increase. Therefore, conformational changes in the extramembrane segments induced by the interaction with ligands can be reflected in the current levels.

1. Introduction. – Many natural ion channels and receptors are composed of multiple protein subunits. For example, the nicotinic acetylcholine receptor channel consists of five subunits [1]. Self-assembly of these subunits form the quaternary protein structures in the membranes to achieve its function. The quaternary structure of this protein has been elucidated from its electron-microscopic investigations [2]. However, details are not known about the methods of the mutual recognition and assembly control of these subunits. Each subunit has a rather large extramembrane domain, and this domain is probably involved in the structural formation of the protein. On the other hand, there are many reports on the self-assembly of amphiphilic peptides, forming pores in the membranes that work as ion channels (for reviews, see [3–6]). It often happens that the self-assembly of channel peptides does not yield a single aggregation number or association state of the peptide, and this eventually produces multiple conductance states which suggest the lack of control of the assembly. By the addition of suitable extramembrane segments, the assembly states of channel-forming peptides in the membrane may be controlled. Our motivation in designing alamethicin-based channel systems is to examine the effect of the extramembrane segments for the assembly control of channel peptides. Although there are several reports regarding the construction of artificial ion channels, very few reports have shed light on the functional roles of the extramembrane segments. In this review, we propose that extramembrane segments can play a role as critical functional units for the control of the assembly states of channel-forming peptides and the eventual channel gating.

2. Modulation of Association States of Channel-Forming Peptides by the Employment of Extramembrane Segments [7]. – It has been reported that a short peptide

derived from a transmembrane segment of the nicotinic acetylcholine receptor-channel protein self-assembled in the membrane forms a channel yielding a channel conductance and kinetics very similar to those of the acetylcholine receptor [8]. It would be surprising that an assembly of a mere *ca.* 20 amino acid peptide can manifest the basic function of the channel pore function of this receptor-channel protein with a molecular mass of *ca.* 290 kDa. Based on this result, it was proposed that, if we employ suitable extramembrane segments, the association control of the pore-forming peptides may be achieved with the help of the extramembrane segments.

A leucine-zipper peptide segment derived from the yeast transcription factor GCN4 was employed (*Fig. 1,a*). This peptide is known to form a stable homo dimer, and the methods of mutual recognition have been well studied [9][10]. A tetraglycine linker has been introduced in this hybrid peptide to connect these segments without disturbing their structural formation as well as to allow ions to enter the channel pore through these two helical segments. As the channel-forming segment, alamethicin was selected. Alamethicins assembled with each other in the membranes to form channels with several conductance states, and this suggests the presence of multiple assembly states or association numbers [11–13]. Various approaches have been reported to create novel channel systems based on this peptide [14–26].

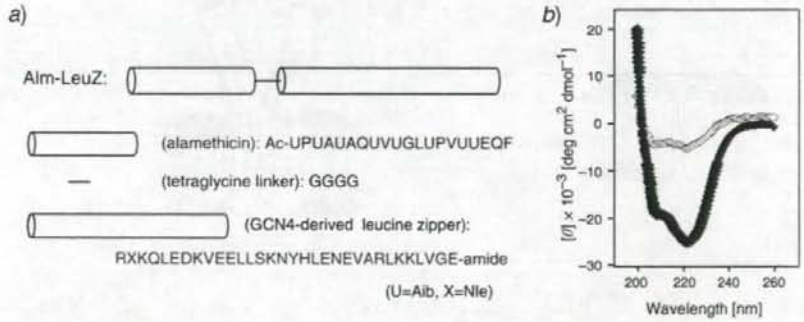

Fig. 1. a) *Design of the hybrid peptide of alamethicin and GCN4 leucine-zipper* (Alm-LeuZ). The amino acids are represented by one-letter codes. U, α-aminobutyric acid (Aib); X, norleucine (Nle). b) *CD Spectra of Alm-LeuZ in 5 mM HEPES* (pH 7.4) (open diamond) *and in the presence of liposomes* (closed diamond) [7].

This peptide was prepared by the solid-phase synthesis and purified by high-performance liquid chromatography (HPLC). The circular dichroism (CD) spectrum of the hybrid peptide in the presence of liposomes suggested that this peptide forms a helical structure in the membrane (*Fig. 1,b*). Interestingly, the helical content of the protein in the absence of liposomes was five times lower than that in the presence of liposomes, suggesting the total helicity of the peptide will increase in the presence of lipid bilayers. Alamethicin is a rather hydrophobic peptide that may form aggregates with other alamethicin segments or may interact with the hydrophobic residues in the GCN4 segment in the aqueous solution. This may produce strain or tension in the helical structure of the alamethicin and GCN4 segments. However, in the presence of

liposomes, alamethicin molecules would more strongly interact with membranes to liberate the GCN4 segment in order to function as the extramembrane segment. This eventually results in the recovery of the respective helical structure of the channel-forming segment in the membrane and the extramembrane segment.

The channel current of alamethicin and the Alm-LeuZ was analyzed according to the planar lipid-bilayer method [27][28] (*Fig. 2*). This method has a sensitivity equal to the patch-clamp technique and can provide the ion flux (channel current) going through a single channel pore formed in real time. The alamethicin without the extramembrane segment-formed channels with several levels of channel conductance at +100 mV (peptide concentration 10 nM; *Fig. 2,a*). In contrast, Alm-LeuZ (50 nM) predominantly formed a channel substantially yielding a single open state (0.12 nS; *Fig. 2,b*). At the higher voltages or higher concentration of the peptide, higher conductance levels were observed (*Fig. 3*). However, even in these cases, the observed channel-open levels are much lower than those of alamethicin itself. This suggests that, by the introduction of the LeuZ extramembrane segment, the association numbers or association states of the alamethicin molecules in the membranes were effectively modulated.

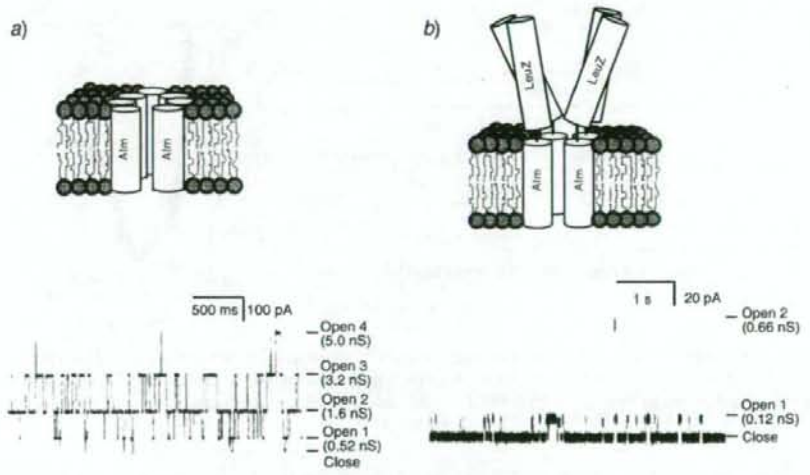

Fig. 2. *Schematic representation of a possible association state and the single channel records of alamethicin amide* (Ac-UPUAUAQUVUGLUPVUUEQF-amide, U = Aib) (*a*) *and Alm-LeuZ* (*b*) [7]. Voltage, +100 mV; electrolyte, 1M KCl.

To obtain information about the association number of Alm-LeuZ, the template-assembled tetramer of Alm-LeuZ (26 kDa) was prepared (*Fig. 4*). The channel conductance most predominantly observed for the template-assembled Alm-LeuZ tetramer (0.12 nS) coincided with that for the self-assembly of Alm-LeuZ (monomer). This suggested that Alm-LeuZ predominantly formed a tetramer in the membranes.

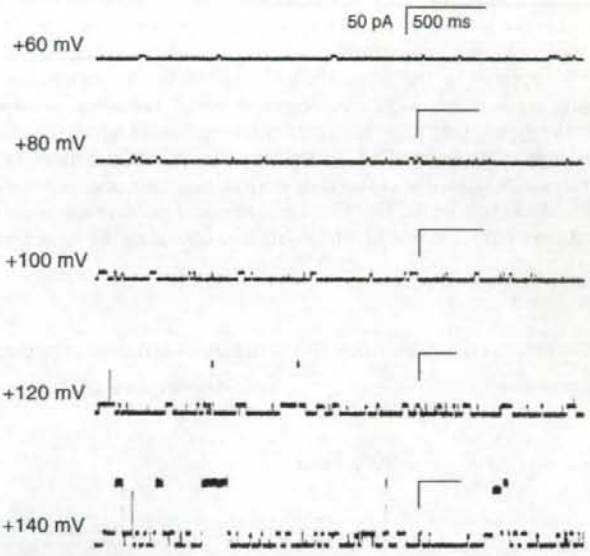

Fig. 3. *Effect of voltage on the channel current of Alm-LeuZ* [7]

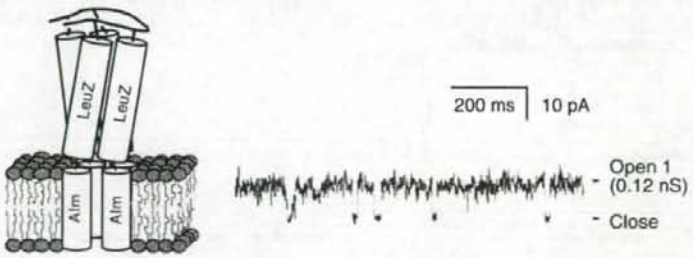

Fig. 4. *Schematic representation and a single channel record of the template-assembled Alm-LeuZ tetramer* [7]. Voltage, +100 mV; electrolyte, 1M KCl.

These results suggested that the introduction of a helical peptide segment significantly affected their assembly states. The GCN4 leucine-zipper is known to have a highly helical conformation. This peptide is known to form a dimer, but not a tetramer. On the other hand, the association of the Alm-LeuZ is effectively modulated to form a tetramer. When the dimers of the GCN4 segments come close together on the membranes, the exchange of the pairs of the GCN4 segments may occur, since the association of these segments should be in equilibrium between the dimers and monomers, and this might eventually result in forming metastable pseudo-tetramer assemblies. As an alternative idea, it may be possible that the strong interaction among

the extramembrane segments may not be crucial for the tetramer formation; steric hindrance among the extramembrane segments may prevent the excess assembly of alamethicin molecules in the membranes to give a predominant association number.

What then happens to the alamethicin assembly in the membrane when the extramembrane segment has a different conformation? Therefore, we designed two analogues of Alm-LeuZ. One peptide has an extramembrane segment derived from the cFos leucine zipper segment [29] (Alm-Fos; *Fig. 5*). The helical content and homodimer formation tendency of this cFos-derived leucine-zipper peptide is reported to be much less than those of the GCN4 leucine zipper. The other one is designed so as to have an extramembrane segment where leucines critical to the helix formation are

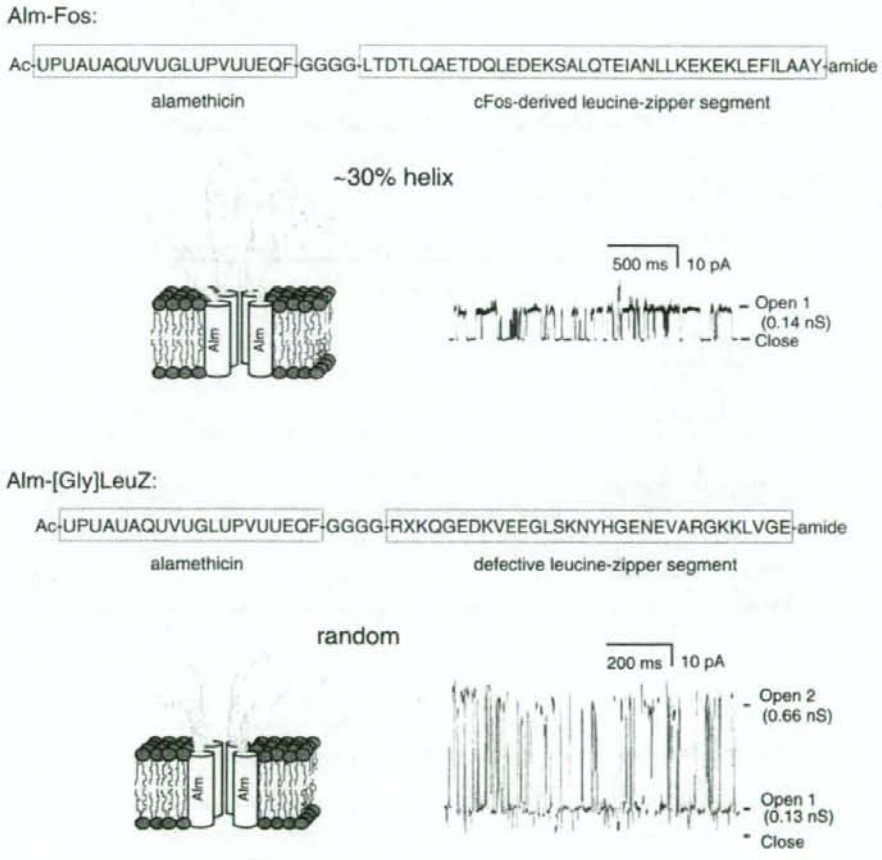

Fig. 5. *Design, schematic representation, and a single channel record of Alm-Fos and Alm-[Gly]LeuZ* (U = Aib, X = Nle) [7]. Voltage, +100 mV; electrolyte, 1M KCl.

substituted with glycines (Alm-[Gly]LeuZ), and this extramembrane segment is expected to have a random structure. Based on the CD analysis, it was confirmed that these extramembrane segments have the desired conformations in the presence of lipid membranes, namely a full helix (Alm-LeuZ), *ca.* 30% helix (Alm-Fos), and no helix (Alm-[Gly]LeuZ). The single channel analysis of these peptides revealed that Alm-Fos predominantly formed channels with a channel conductance (0.14 pS) very similar to that of Alm-LeuZ, whereas, in the case of Alm-[Gly]LeuZ, a higher conductance of 0.66 nS was frequently observed in addition to that of 0.13 nS. This suggested that the decrease in the helical content in the extramembrane segments leads to a higher aggregation number of the peptide or different conformation that produced a higher channel conductance.

3. Reversible Regulation of the Channel Current of Alm-[Ida]Fos by Fe^{III} [30]. – These results suggested the possibility that, if we can employ extramembrane segments that alternate the helical contents by the interaction with extracellular ligands, these can be artificially designed receptor channel systems that output the ligand interaction as changes in the channel current. We have recently shown that the chelation of Fe^{III} with a pair of diiminoacetic acid derivatives of lysine (Ida; *Fig. 6*) [31] at the n and $n+2$ positions yielded a significant helix destabilization when the Ida residues are incorporated into the helical peptides [32]. We then applied these findings to the reversible control of the helical peptide structure and mutual recognition. A hybrid peptide of alamethicin that has the cFos segment with two Ida residues at positions 42 and 44 as the extramembrane segment was designed (Alm-[Ida]Fos; *Fig. 7,a*). The tetraglycine linker was also used here so that these peptide segments work independently, and the ions pass through the spaces between the linkers into the channel pores. For the synthesis of this peptide, we employed a novel method of preparing the Ida-containing peptides in which the lysine (Lys) residues are converted into Ida residues on the solid-phase resin [32]. The CD spectra of Alm-[Ida]Fos in the presence of liposomes suggested that Alm-[Ida]Fos formed a helical structure (*Fig. 7,b*). The addition of Fe^{III} led to a decrease in the helical content of Alm-[Ida]Fos, indicating the destabilization of the [Ida]Fos extramembrane segment by Fe^{III}.

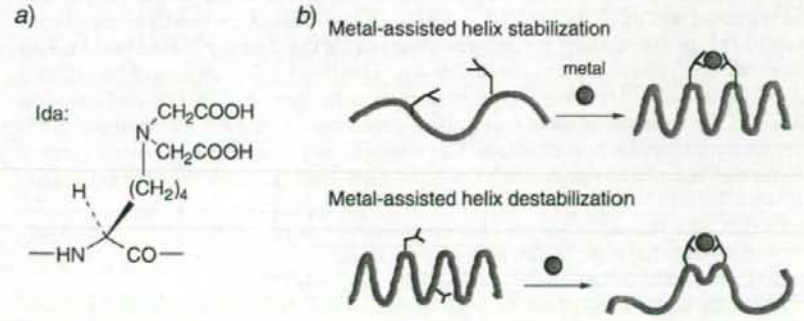

Fig. 6. *Structure of Ida* (*a*), *and concepts of metal-assisted helix stabilization and destabilization* (*b*) [32]

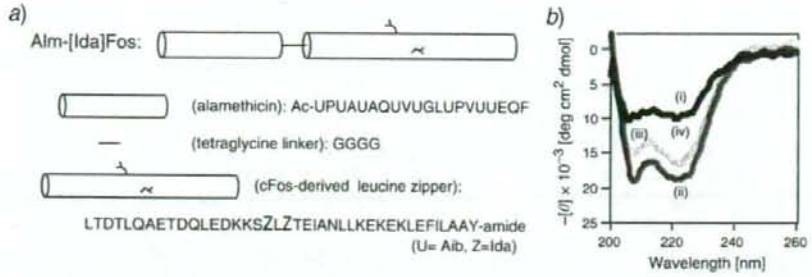

Fig. 7. *Design* (*a*) *and CD spectra* (*b*) *of the Alm-Fos peptide containing Ida residues* (Alm-[Ida]Fos) *in the absence* (*i*) *and presence* (*ii*) *of liposomes with 5 and 10 equiv. of Fe(III)* (*iii* and *iv*, resp.) [30]

We next examined whether the channel current of Alm-[Ida]Fos can be modulated by Fe^{III}. Self-assembly of the Alm-[Ida]Fos in the membrane formed channels, and the predominant channel conductances in the absence of Fe^{III} at +150 mV were 0.13 and 0.72 nS. These current levels were substantially identical to those for Alm-Fos without possessing the Ida residues [7] under the same conditions. This suggested that the introduction of Ida did not produce a crucial effect on the channel conductance of the Alm-Fos peptides.

Next, we examined the effect Fe^{III} on this channel system. Upon addition of 2 μM Fe^{III}, a 20-fold increase in the channel current was observed (*Fig. 8*). This suggests that the conformational change in the extramembrane segment induced by the recognition with Fe^{III} is effectively transmitted as the channel current. The process of this Fe^{III}-mediated channel-current modulation was reversible. Upon addition of the excess amount of EDTA (final concentration 10 μM), the channel current decreased to the original level in the absence of Fe^{III}. This basically identical tendency was observed during the repetitive addition of Fe^{III} and EDTA, and the channel currents at 15–30 pA were observed during the absence of Fe^{III}, and 330–400 pA in its presence. Therefore, the addition of Fe^{III} produced more than a 10–20 times increase in the channel current. To confirm whether Fe^{III} itself does not contribute to the channel-current control, similar experiments were conducted with Alm-Fos, a peptide which has the same amino acid sequence as Alm-[Ida]Fos, but lacks the Ida residues. As a result, no significant effect of Fe^{III} on the channel current was observed for the channel formed by Alm-Fos. Therefore, the channel current increase of Alm-[Ida]Fos by the addition of Fe^{III} should not be attributed to the membrane perturbation by Fe^{III}, but to the conformation change in the extramembrane segment by interaction with Fe^{III}. An artificial Fe^{III}-receptor ion channel is thus established. In addition, there are several reports regarding the creation of artificial ion channels that have a sensing function of the external ligands [6][14][15][21][33–38]. However, most of them have been designed so that the interaction with ligands plugs the channel pores and stops the channel current. Only very few of them have the ability to detect the ligand as a membrane current increase, as usually observed in natural ligand-gated ion channels [37][38].

As for the mechanism of this gating control, one possible explanation is that, as was suggested in our study of Alm-LeuZ, the assembly of the alamethicin–leucine zipper

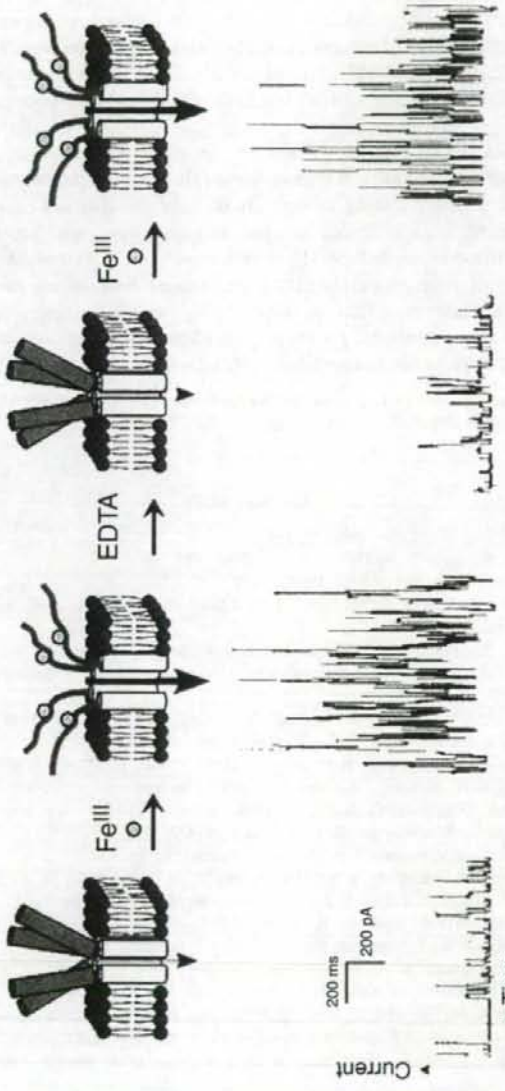

Fig. 8. *Schematic representation and channel-current records of the Alm-1/da1Fos channel.* Conformation change in the Ida-containing extramembrane segments by the interaction with Fe^{III} produced an increased channel current [30].