Translocation of Analogues of the Antimicrobial Peptides Magainin and Buforin across Human Cell Membranes*

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Cationic antimicrobial peptides play important roles in innate immunity. Compared with extensive studies on peptide-bacteria interactions, little is known about peptide-human cell interactions. Using human cervical carcinoma HeLa and fibroblastic TM12 cells, we investigated the cellular uptake of fluorescent analogues of the two representative antimicrobial peptides magainin 2 and buforin 2 in comparison with the representative Arg-rich cell-penetrating Tat-(47–57) peptide (YGRKKRRQRRR). The dose, time, temperature, and energy dependence of translocation suggested that the three peptides cross cell membranes through different mechanisms. The magainin peptide was internalized within a time scale of tens of minutes. The cooperative concentration dependence of uptake suggested that the peptide forms a pore as an intermediate similar to the observations in model membranes. Furthermore, the translocation was coupled with cytotoxicity, which was larger for tumor HeLa cells. In contrast, the buforin peptide translocated within 10 min by a temperature-independent, less concentration-dependent passive mechanism without showing any significant cytotoxicity at the highest concentration investigated (100 μM). The uptake of the Tat peptide was proportional to the peptide concentration, and the concentration dependence was lost upon ATP depletion. The peptide exhibited a moderate cytotoxicity at higher concentrations. The time course did not show saturation even after 120 min. The buforin peptide, covalently attached to the 28-kDa green fluorescent protein, also entered cells, suggesting a potency of the peptide as a vector for macromolecular delivery into cells. However, the mechanism appeared to be different from that of the parent peptide.

Cationic antimicrobial peptides play important roles in innate immunity (1–4). Broad antimicrobial spectra, highly selective toxicity, as well as difficult resistance development make these compounds promising candidates as novel antibiotics for clinical use. Intensive investigations on peptide-bacteria interactions have shown that some peptides kill bacteria mainly by permeabilizing the membranes, whereas others target intracellular compounds, such as nucleic acids, although recent studies have suggested that many peptides possess both modes of action (2, 4, 5).

Magainin 2 (Table I) isolated from the skin of the African clawed frog Xenopus laevis is a representative membrane-acting peptide composed of 23 amino acid residues (6–8). Addition of the peptide to bacterial cells immediately induces the permeabilization of both outer and inner membranes, leading to cell death (9, 10). A fluorescent magainin peptide mainly associates with bacterial membranes and does not enter cells (10), although native magainin 2 was recently reported to enter cells (11). An example of the peptides targeting intracellular substances is buforin 2, which was discovered in the stomach of the Asian toad Bufo bufo gargarizans (12). A fluorescent buforin 2 rapidly enters bacterial cells without perturbing the barrier properties of the membranes, binding to DNA and RNA (10).

On the other hand, interactions of cationic antimicrobial peptides with human cells are not well characterized. The information on cell penetration available is limited to dermaseptins (13–15), PR-39 (16), and protegrin 1 (17), although many investigators examined hemolytic activity (9, 18) and cytotoxicity (19–22). In contrast, short Arg-rich cationic peptides have recently been shown to enter mammalian cells by an energy (ATP)-independent, non-endocytotic pathway and have been utilized as vectors for delivering membrane-impermeable drugs, oligonucleotides, and proteins into cells (23). Model membrane studies revealed that the membrane-acting magainin peptide also translocates across lipid bilayers (24, 25), although less effectively than buforin 2 (18). Therefore, cationic antimicrobial peptides, including those of the membrane-permeabilizing class, can penetrate human cells. However, although this issue has rarely been investigated, it is important for the development of novel peptides for therapeutic use as well as for understanding the roles of antimicrobial peptides in innate immunity.

In this study, we investigated the translocation of fluorescent-labeled analogues of magainin 2 and buforin 2 across human cell membranes in comparison with the Tat-(47–57) peptide (Table I), a representative Arg-rich cell-penetrating peptide. Both tumor (human cervical carcinoma HeLa) and normal (human fibroblastic TM12, Ref. 26) cells were used because magainin peptides are known to exhibit selective toxicity for tumor cells (19–21, 27). The dose, time, temperature, and energy dependence of translocation suggested that the three peptides crossed cell membranes through different mechanisms. Furthermore, the buforin peptide, covalently attached to the 28-kDa green fluorescent protein also entered cells, suggesting a potency of the peptide as a vector for macromolecular delivery into cells.

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EXPERIMENTAL PROCEDURES

Materials—The reagents for peptide synthesis were purchased from Agape BioTechnology (Foster City, CA). All fluorescent probes. Peptide labeling were obtained from Molecular Probes (Eugene, OR). Dulbecco’s modified Eagle’s medium (DMEM) was supplied by nacalai tesque (Kyoto, Japan). Bis(sulfosuccinimido)suberate (BS3) linker and MTT were purchased from Pierce and Sigma, respectively. GFP was supplied by Upstate Biotechnology (Lake Placid, NY). All other chemicals from Wako (Tokyo, Japan) were of special grade.

Peptide Synthesis—The peptide was synthesized by a standard fluorogenic 9-ylation (Fmoc)-based solid phase method on an Applied Biosystems Model 433A peptide synthesizer, as previously described (28). Fluorescent labeling of the peptides was performed as follows. After cleavage from the resin, the Cys residue of the magainin peptide, the buforin analogue dissolved in 5 mM phosphate buffer (pH 7.4) was conjugated with thiol-reactive Texas Red(R) C2, maleimide in acetonitrile/methanol (1:1, v/v). The N terminus of the Tat peptide on the resin was labeled with Texas Red(R)-X succinimidy ester in N,N-dimethylformamide and isopropanol, washed with N,N-dimethylformamide and acetonitrile, and then cleaved from the resin. The purity of the synthesized peptides was determined by analytical reversed-phase high performance liquid chromatography (RP-HPLC) and ion spray mass spectrometry.

Cell Culture—HeLa and TM12 cells were cultured in DMEM with 10% fetal bovine serum (FBS). The cells were maintained under 5% CO2 at 37°C.

Cellular Uptake of Peptide—The cells (1 \times 10^5/cm\(^2\)) were plated on 24-well microplates (2 cm\(^2\)/well), and then incubated overnight. After removal of the medium, the cells were exposed to peptide solutions (200 \muM) freshly dissolved in DMEM with 10% FBS for various periods at 37°C, unless otherwise indicated. The peptide solutions were then aspirated, and the cells were washed three times with phosphate-buffered saline (PBS) of pH 7.4 and lyzed with 0.5 ml of 10% SDS in PBS. The protein content of the cells was determined by the Bio-Rad DC protein assay kit (Hercules, CA). The internalized Texas Red-labeled peptides were fluorometrically quantified with a Shimadzu RF-5300 spectrofluorometer at an excitation wavelength of 583 nm and an emission wavelength of 610 nm.

Effects of Low Temperature or Sodium Azide—The assays were performed as described above with the exception that the cells were preincubated for 30 min either at 4°C or in the presence of 0.5% (w/v) sodium azide in DMEM with 10% FBS at 37°C. The subsequently added peptide solution was cooled at 4°C or contained 0.5% (w/v) sodium azide.

Confocal Laser Microscopy—The cells (1 \times 10^5/cm\(^2\)) were plated on 8-well chamber slides (1 cm\(^2\)/well), incubated overnight, and then treated with peptide solutions as described above. For nuclear staining, 5 \muM SYTO16 (Molecular Probes) was added to the peptide solutions. After washing three times with PBS, the cells were fixed with 4% (w/v) paraformaldehyde in PBS for 5 min at room temperature, washed again with PBS, and mounted in a glycerol/PBS (8:1, v/v) solution containing 0.1% p-phenylendiamine dihydrochloride. Microscopic examination was performed on a Bio-Rad MRC1024 confocal laser microscope.

Cytotoxicity—The cells (5 \times 10^5 cells/well) were cultured in 96-well microplates and incubated overnight in DMEM containing 10% FBS. After removal of the medium, the cells were incubated with 100 \muM of peptide dissolved in the medium for 24 h at 37°C. Ten microliters of a 5 mM MTT solution in PBS was then added, and the cells were further incubated for 5 h. Thereafter, the precipitated MTT was dissolved overnight in 0.1 M sodium hydrosulfite in PBS for 0.1 M HCl. The percent cell viability was determined by 100 \times (OD\(_{570}\) – OD\(_{550}\)) for peptide-treated cells/OD\(_{550}\) for untreated cells. OD\(_{570}\) and OD\(_{550}\) determined at 595 and 655 nm.

Buforin-GFP Conjugate—The N terminus of the buforin peptide dissolved in sodium bicarbonate buffer (pH 8.3) was labeled with Rhodamine Red-X succinimidyl ester in dimethylsulfoxide. Minor byproducts were removed by RP-HPLC. The labeled peptide (65 \muM) and GFP (24 \muM) were mixed in PBS (pH 7.4). 30-fold excess over the protein of the amino group-reactive linker BS3 freshly dissolved in sodium phosphate buffer (pH 5.0) at a concentration of 10 mM was added to the mixture solution. After 30 min, the reaction was stopped with excess 1 M Tris.

### Table 1

| Peptide | Sequence \(^a\) | Charge \(^b\) |
|---------|----------------|------------|
| Magainin 2 | GIGKFLHSAKKFKGAFVGEIMNS | +3 |
| MG2d | GIGKFLHSAKKFKGAFVQIMNC | +5 |
| Buforin 2 | TRSSRAQLGQPVGRHVRLLRK | +7 |
| BF2d | TRSSRAQLGQPVGRHVRLLRKGGC | +7 |
| Tat (47–57) | Texas Red-YGKKRQRRR | +8 |

\(^a\) The underlined residues show differences from the original peptide.  
\(^b\) Approximate charge at physiological pH (7.4).

The aggregates were precipitated by centrifugation, and then the unreacted reagents in the supernatant were removed from the buforin-GFP conjugate by Millipore Micron centrifugal filter devices (Bedford, MA). The average number of attached buforin peptide molecules per GFP was 1.77, as estimated by UV-visible absorption.

RESULTS

Peptide Design—The magainin 2 derivative (MG2d, Table I) was designed as follows. The translocation occurs upon the disintegration of a pore (24). The translocation efficiency is known to be enhanced by increasing the peptide’s positive charge, which destabilizes the intermediate pore (29). Therefore, the Glu19 residue of magainin 2 was replaced with Gln, and the C terminus was amidated. This modification increased the charge by +2. Texas Red, which is brighter and has a lower background than other commonly used red fluorescent dyes and is suited to laser excitation, was linked to the Cys residue introduced at the 23rd position. The F12W substitution was for easy quantitation of the unlabeled peptide by optical density at 280 nm. The Trp introduction to the buforin 2 analogue BF2d was for the same reason. The C-terminal extension GCC was added to link various molecules via the SH group in future studies. In this study, Texas Red was covalently attached to the peptide for laser microscopy. The Tat-(47–57) peptide includes the sequence responsible for the cellular uptake of the human immunodeficiency virus-1 Tat protein consisting of the polycationic region 49–57 (30). The N terminus of the peptide was also labeled with this dye. The labeling position had no effect on peptide-cell interactions: We also synthesized a BF2d analogue with the dye at the N terminus. The concentration and time dependence of peptide uptake were almost identical to those of BF2d (data not shown).

Intracellular Distribution—The fluorescent peptides (5 \muM) were incubated with HeLa or TM12 cells for 30 min at 37 or 4°C, and the intracellular distributions of the peptides were examined by confocal laser microscopy. At 37°C, MG2d translocated across the cell membranes of both cells (Fig. 1, A and D) and was almost uniformly distributed inside the cells except for the nuclei in which the peptide was less concentrated. This could be more clearly seen by co-labeling with the nuclear-staining dye SYTO16 (Fig. 1B). Similar results were also obtained at 4°C (Fig. 1C). In contrast to the magainine analogue, the intranuclear concentrations of BF2d (Fig. 1E) and Tat-(47–57) (Fig. 1H) were similar to those in the cytosol. The co-labeling with SYTO16 clearly indicated that BF2d had entered the nuclei (Fig. 1F). The uptake of these peptides was also not significantly temperature-dependent (Fig. 1, G and I). The treatment of MG2d roughened the cell surfaces compared with the other two peptides.

To prove that most peptide molecules were not surface-bound but internalized, we performed the optical sectioning of HeLa cells treated with MG2d as in Fig. 1A. The focus plane was changed from bottom to top in the vertical axis range of 32.8 \muM at an interval of 1.64 \muM. Representexative photographs are shown in Fig. 2. Texas Red fluorescence was only observed in the central region of the cell.
Concentration Dependence—In addition to fluorescence microscopy, quantitative data on the cellular uptake of the peptides are useful for the elucidation of translocation mechanisms. Fluorescent flow cytometry has often been utilized for this purpose (30). An alternative, more quantitative method was used in this study. The amount of peptide taken up was fluorometrically determined after cell solubilization. At the same time, the amount of cellular proteins was quantitated as a measure of the number of cells. The amount of peptide uptake per mg of cellular protein during a 30-min incubation at 37 °C is plotted as a function of peptide concentration for HeLa and TM12 cells in Fig. 3, A and B, respectively. Although these values include the amount of peptide bound to the cell surface, it is clear from Figs. 1 and 2 that most peptide molecules were internalized.

The uptake of MG2d into both cells was apparently cooperative with respect to peptide concentration (Fig. 3, circles). The double logarithmic plots gave slopes of 1.6 (r = 0.982) and 1.4 (r = 0.995) for HeLa and TM12 cells, respectively (plots not shown). In contrast, the uptake of BF2d was much less concentration-dependent (Fig. 3, squares). The Tat-(47–57) peptide was incorporated into the cells almost linearly to the peptide concentration of at least 15 μM (Fig. 3, triangles).

Effects of Low Temperature and Metabolic Inhibitor—Endocytic processes are known to be inhibited at lower temperatures or by ATP depletion (31–33). Open bars in Fig. 4A show the peptide uptake by HeLa cells at 4 °C relative to that at 37 °C (control). The uptake of MG2d was reduced by 40% whereas those of BF2d and Tat-(47–57) were insensitive to temperature. Shaded bars in Fig. 4A show the effects of sodium azide, which blocks ATP synthesis by inhibiting cytochrome oxidase, on peptide uptake. The internalization of MG2d was slightly (~20%) reduced by the metabolic inhibitor while that of BF2d was not affected by the treatment. In contrast, the azide treatment largely abolished the concentration depend-
practically non-toxic at least up to 100 TM12 cells (\(n = 3\)). In contrast, BF2d was a candidate as a vector for delivering macromolecules into cells. The peptides (5 \(\mu\)M) were incubated with HeLa cells for 30 min at 37 or 4 °C. The amount of peptide uptake was fluorometrically determined after washing and solubilization. Open bars show peptide uptake at 4 °C relative to that at 37 °C. To examine the effects of sodium azide, HeLa cells were preincubated with 0.5% (w/v) \(\text{Na}_{2}\text{SO}_{4}\) and the uptake experiments were performed in the presence or the absence of the metabolic inhibitor for 30 min at 37 °C. The peptide concentration was 5 \(\mu\)M. Shaded bars show peptide uptake with \(\text{NaN}_3\) relative to that without \(\text{NaN}_3\). For Tat-(47–57), similar experiments were carried out at various peptide concentrations. Open and closed triangles show peptide uptake without and with \(\text{NaN}_3\), respectively. Each datum expresses average ± S.D. (\(n = 3\)).

The absence of any significant presence of Tat uptake (Fig. 4B). Such a phenomenon was not observed for BF2d (data not shown).

**Time Dependence**—Fig. 5, A and B show the time dependence of peptide uptake by HeLa and TM12 cells, respectively. For MG2d (circles), the uptake kinetics was biphasic. A 5-min incubation was sufficient for the half-maximal uptake. A longer incubation gradually increased uptake, which was saturated at 60–120 min. The time course of BF2d internalization was completely different (squares). The uptake was rapid and reached the maximum at 10 min, and then gradually decreased. In contrast to these antimicrobial peptides, the Tat-(47–57) peptide was continuously taken up at least up to 120 min (triangles).

**Cytotoxicity**—The cytotoxicities of the peptides without Texas Red labeling were evaluated by the standard MTT assay, which demonstrates active energization of cells and is conventionally used as a measure of cell viability. The percent viability is plotted as a function of the peptide concentration in Fig. 6. In the presence of MG2d, the cell viability was abruptly decreased beyond 10 and 20 \(\mu\)M for HeLa (open circles) and TM12 cells (closed circles), respectively. In contrast, BF2d was practically non-toxic at least up to 100 \(\mu\)M for both cells (squares). Upon Tat-(47–57) treatment (triangles), the cell viability was decreased as a function of peptide concentration over the range of 0.1 to 100%.

**Buforin-Protein Conjugate**—The absence of any significant cytotoxicity suggests that the buforin peptide is a promising candidate as a vector for delivering macromolecules into cells. To examine this possibility, BF2d was conjugated with GFP (28 kDa) by use of the bifunctional cross-linker BS². Fig. 7A shows that GFP alone did not enter HeLa cells. In contrast, the conjugate was internalized into the cells, as confirmed by both GFP (protein part) and Texas Red (peptide part) fluorescence (Fig. 7B). The conjugate was uniformly distributed within the cells and reached the nuclei, similarly to the vector peptide (Fig. 1E). The uptake efficiency was, however, lower than that of BF2d alone (Fig. 7D) and diminished at low temperature (Fig. 7E) or in the presence of sodium azide (Fig. 7F).

**DISCUSSION**

**Magainin Analogue**—Interactions of magainins with lipid bilayers are well characterized (for review, see Refs. 7 and 8). The peptides preferentially bind to negatively charged phospholipids, forming amphipathic helices (28). Their affinities for mammalian cells, on the surface of which anionic phospholipids are not exposed, have been considered to be low, although weak hydrophobic interaction drives peptide binding (34). The amounts of MG2d taken up by both cells were larger than those of BF2d and Tat-(47–57) (Fig. 3), despite having the smallest positive charge. The hydrophobicity of MG2d, which has several hydrophobic residues (2Ile, 2Phe, Trp, Leu, Val), was the largest among the three peptides (Table I).

After membrane binding, several magainin helices form a toroidal pore (diameter 2–3 nm) with surrounding lipid molecules in artificial membranes (35). Upon pore disintegration, a fraction of the peptide molecules translocates across the membrane (24). This is reflected by a cooperativity in peptide binding (22). The cooperativities observed in cellular uptake of MG2d (Fig. 3) and cytotoxicity (Fig. 6) suggest that the magainin derivative also forms a pore in cell membranes and that pore formation and subsequent cell entry are closely related to cell death. One possibility is that membrane permeabilization leads to cell death by dissipating membrane potential and leakage of intracellular molecules. Another possibility is that the peptide damages mitochondria (36), resulting in apoptosis. The surface-roughening effect of MG2d (Fig. 1) also suggests membrane activity of this peptide.

In studies using large unilamellar vesicles of 100 nm in diameter, the pore formation-translocation process is biphasic and reaches pseudo-equilibrium within ~10 min (24). In cells that are much larger and have various organelles and macromolecules that the magainin peptide can interact with, the internalization takes much longer, consistent with the kinetic uptake data (Fig. 5). The uptake of MG2d was partially inhibited at low temper-
alone, BF2d alone were incubated with HeLa cells for 30 min at 37°C as examined by confocal laser microscopy.

or 4°C indicates that the membrane permeation occurs by a mechanism across model lipid bilayers without perturbing lipid organization elucidated. The observation that peptides also translocate cell membranes (10), although the mechanism has not yet been elucidated. This is not clear at present. ATP depletion can cause various changes in cell membranes, including the inhibition of endocytosis-mediated recycling of cell surface molecules (33), the blockage of transbilayer movement of phospholipids (41), and the dissipation of transmembrane potential (42). The partial reduction (20%) of MG2d uptake by the metabolic inhibitor (Fig. 4B) suggests some involvement of energy-dependent processes. At lower peptide concentrations, the presence of sodium azide induced a slight enhancement in uptake. The reason for this is not clear at present. ATP depletion can cause various changes in cell membranes, including the inhibition of endocytosis-mediated recycling of cell surface molecules (33), the blockage of transbilayer movement of phospholipids (41), and the dissipation of transmembrane potential (42). The partial reduction (20%) of MG2d uptake by the metabolic inhibitor (Fig. 4B) may be related to these alterations in the cell surface.

CONCLUSION

We investigated the cellular uptake of the analogues of the two representative antimicrobial peptides, membrane-acting magainin 2 and nuclear targeting buforin 2, in comparison with the Tat-(47–57) peptide. Together with previous reports on
various cell-penetrating peptides, our data show the existence of at least three mechanisms for cell entry. 1) The magainin-class peptide crosses the cell membranes by the transient pore-formation-translocation mechanism that can be reproduced in artificial lipid bilayer systems (7, 8). The process is moderately temperature-sensitive. 2) The buforin class peptide translocates into the cells without perturbing the cell membranes by an unknown mechanism. The translocation phenomenon is also observable in artificial systems (18). The process is not influenced by temperature or a metabolic inhibitor, and the cytotoxicity is minimal. 3) The Tat-class peptide enters the cells by an unknown mechanism that many Arg-rich peptides may share (38). The temperature and energy dependence vary among the peptide sequences and the cell lines used. The peptides show moderate cytotoxicities at higher concentrations (40). The membrane permeation cannot be reproduced in liposomal systems. 2 It is not clear whether the Antennapedia peptides (penetratins) (43) belong to this category or not because conflicting results were reported on the translocation across lipid bilayers (44, 45). It should be noted that the attachment of a cargo molecule can change the translocation mechanism, as observed for BF2d-GFP.

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