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The Membrane-Lytic Peptides K8L9 and Melittin Enter Cancer Cells via Receptor Endocytosis following Subcytotoxic Exposure

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SUMMARY

We investigate the cell entry mechanism of the membrane-lytic peptides K8L9 and melittin in cancer cell lines. K8L9 and melittin interacted with the highly expressed endocytic receptors neuropilin-1, low-density lipoprotein-related protein receptor 1 (LRP1), and transferrin receptor. Silencing of these receptors by small interfering RNAs (siRNAs) attenuated the cytotoxic activity of K8L9 in four cancer cell lines. Intracellular K8L9 and melittin triggered enlargement of the lysosomal compartments and cytosolic translocation of cathepsin B. Hsc70 was identified as a melittin-interactive molecule using coimmunoprecipitation and mass spectrometry, and Hsc70-siRNA attenuated the cellular uptake of K8L9 and cytotoxic activity by K8L9 and melittin. These findings suggest that K8L9 and melittin can enter cancer cells via receptor endocytosis following subcytotoxic treatment and subsequently affect lysosomal compartments.

INTRODUCTION

Antimicrobial peptides (AMPs) play an important role in host defense against bacterial pathogens. AMPs are naturally occurring peptides found in plants, animals, bacteria, fungi, and synthetic peptides (Zasloff, 2002; Shai and Oren, 2001). Many cationic AMPs are unstructured in solution, but can form α-helical or β strand secondary structures upon contact with the cell membrane. Concentrated cationic moieties on molecules with a strong amphipathic structure bind to lipid headgroups of the anionic microbial cell membrane while the nonpolar side is exposed to the membrane acyl core and disrupts the membrane bilayer by forming pores (Zasloff, 2002; Brogden, 2005). Different models have been proposed to account for the mechanism of membrane disintegration induced by membrane-lytic peptides (MLPs), namely the barrel stave, toroidal pore, and detergent-like models (Hoskin and Ramamoorthy, 2008). Some AMPs exhibit cytotoxic activity against cancer cells, whose outer membrane contains 3% to 9% negatively charged phosphatidylserine, which is slightly higher than that in normal cells (Papo and Shai, 2003).

Several naturally occurring AMPs that exhibit anticancer activity have been identified, for example, melittin from the venom of honeybees, magainins from the skin of the African clawed frog, and buforins from the stomach of the Korean toad (Zasloff, 2002; Hoskin and Ramamoorthy, 2008). These AMPs not only disrupt the cell membrane but can also enter the cytoplasm and affect various intracellular targets. Melittin stimulates phospholipase A2 and D and reduces the activity of IκB kinase (Park et al., 2007; Lee et al., 2001). Magainin 2 and buforin 2 exert cytotoxic activity against cancer cells without damaging cell membranes by disrupting mitochondrial functions (Cruz-Chamorro et al., 2006; Lee et al., 2008). Histatin 5, found in human salivary secretions, and ApoEdpL-W (human ApoE apolipoprotein derivative) exert antifungal activity in *Candida albicans* via endocytosis (Mochon and Liu, 2008; Rossignol et al., 2011).

Cationic membrane-active peptides (MAPs) include not only AMPs, but also amyloid β peptide and cell-penetrating peptides (CPPs) (Last et al., 2013). Some MAPs are known to enter the cell via endocytosis. Amyloid β peptide enters the cytoplasm via low-density lipoprotein receptor-related proteins (LRPs) and the receptor for advanced glycation end products (RAGEs) and subsequently disrupts mitochondrial functions (Takuma et al., 2009; Deane et al., 2004). It is reported that stearylated K6L9, an MLP, delivers plasmids into cells by clathrin- and caveolin-mediated endocytosis (Zhang et al., 2013a).

We recently showed that the lytic peptide K8L9 can enter the cytoplasm via endocytosis-dependent or -independent pathways (Ohara et al., 2013). We hypothesized that some MLPs might interact with receptors and enter cells via endocytosis in a similar fashion to the peptides mentioned above. In this study, we demonstrate that K8L9 and melittin interact with receptors and enter the cytoplasm via endocytosis. We try to identify the molecules with which artificial and natural peptides, K8L9 and melittin, respectively, interact to achieve cellular entry, and we analyze the intracellular trafficking of the peptides.

RESULTS

K8L9 Not Only Disrupts Cell Membranes from the Outside, but Also Enters the Cytoplasm via Endocytosis following Subcytotoxic Exposure

We previously showed that endocytosis inhibitors attenuate the cytotoxic activity and cellular uptake of the lytic peptide K8L9 in U251 cells. To investigate this phenomenon in other human

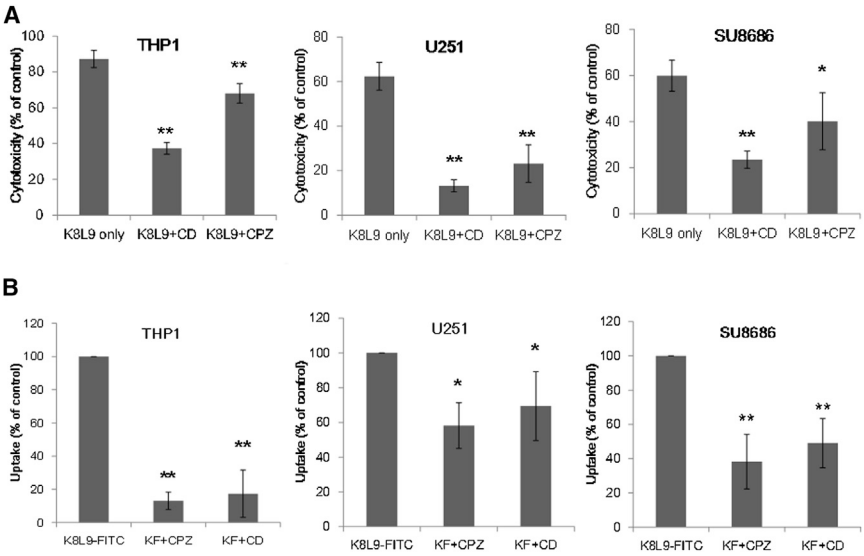

Figure 1. K8L9 Not Only Disrupts the Cell Membrane from the Outside, but Also Enters the Cytoplasm

(A) Effect of endocytosis inhibitors on K8L9 cytotoxicity. Cells were pretreated with the actin polymerization inhibitor cytochalasin D (CD, 0.75–1.00 μM) or the clathrin inhibitor chlorpromazine (CPZ, 10 μM) for 1 hr prior to incubation with 10–20 μM K8L9 for a further 24 hr. The results are represented as the means ± SD (bars) of three or four independent experiments. *$p < 0.05$, **$p < 0.01$.

(B) Effect of endocytosis inhibitors on cellular uptake of K8L9-FITC peptide. Cellular uptake of K8L9-FITC (KF) was determined by flow cytometry. The bar charts indicate the mean fluorescence intensity (MFI) after normalization to cells incubated with K8L9-FITC alone. The results are represented as the means ± SD (bars) of three independent experiments. *$p < 0.05$, **$p < 0.01$.

cancer cell lines, we used the acute monocytic leukemia and pancreatic cancer cell lines THP1 and SU8686, respectively. The viability of cells treated with K8L9 increased after pretreatment with the actin polymerization inhibitor cytochalasin D or the clathrin inhibitor chlorpromazine (Figure 1A). On the other hand, cytotoxic activity of L-K8L9 containing all L amino acids was weakly inhibited by actin polymerization inhibitor compared with that of K8L9 (Figure S1 available online). Pretreatment of cells with cytochalasin D or chlorpromazine decreased the cellular uptake of K8L9-fluorescein isothiocyanate (FITC) (Figure 1B). These results suggest that K8L9 not only disrupts the cell membrane from the outside, but also enters the cytoplasm via endocytosis following subcytotoxic exposure.

Membrane Attack by K8L9 Is Moderate Compared with that by K6L9 and Melittin

To create a molecular-targeted anticancer drug with a novel mode of action, we designed the lytic peptide K8L9, which moderately attacks cell membranes compared with conventional membrane disrupting peptides (Kohno et al., 2011). Since we hypothesized that the moderate membrane attack activity of K8L9, when compared with more potent MAPs, facilitates its cellular uptake via endocytosis, we assessed the concentration and time-dependent cytotoxic activity of melittin, K6L9, and K8L9. Cytotoxicity was observed in cells treated for 24 hr with 5 μM melittin and 7.5 μM K6L9, but not in cells treated with 10 μM K8L9 (Figure 2A; Table S1). K8L9 has antimicrobial activity against *E. coli.*, and its IC_{50} value against JM109 was 2.9 ± 0.21 μM (Figure S2).

We proceeded to compare the membrane attack activity of the three peptides in U251 and H460 cells. Cells were treated with 30 μM K8L9, 10 μM K6L9, or 5 μM melittin for 1 hr or 24 hr. In 1 hr exposure, K8L9 showed less cytotoxic activity when compared with melittin and K6L9 (Figure 2B). The cytotoxic activity and cellular uptake of melittin were only weakly attenuated by endocytosis inhibitors compared with K8L9 (Figure S3). These results suggest that it is easier to detect K8L9 cellular uptake via endocytosis because it only moderately disrupts cell membranes when compared with more potent MAPs.

Interaction of K8L9 and Melittin with Endocytic Receptors

It has previously been reported that amyloid β peptide enters cells via LRP1 and RAGE (Takuma et al., 2009; Deane et al., 2004) and that C end rule peptide, containing a C-terminal arginine/lysine, enters cells via neuropilin 1 (NRP1) (Teesalu et al., 2009). These receptors interact with the cationic peptides and translocate them to the intracellular space via endocytosis. We focused on highly expressed endocytosis receptors in cancer cell lines and examined whether K8L9 and melittin could interact with them. NRP1 and LRP1 were highly expressed in pancreatic cancer and glioma cell lines SU8686 and U251, respectively, whereas transferrin receptor (TfR) was highly expressed in lung cancer H460 and breast cancer T47D cell lines (Figure 3A). We investigated the interaction of these receptor on K8L9 and melittin by coimmunoprecipitation and western blotting using biotinylated K8L9 or melittin and NeutrAvidin resins. In the presence of melittin-biotin, clear interaction bands were observed for NRP1 and TfR, but no bands were observed in the presence or absence of K8L9-biotin (Figure 3B).

To compare the binding affinity of cationic peptides to NRP1, the binding of K8L9, melittin, and magainin 2 to immobilized NRP1 on the surface of sensor chips was monitored using a Biacore analyzer. While NRP1 was not coimmunoprecipitated with K8L9 but with melittin, increment of the concentration of K8L9 and melittin clearly increased their binding to NRP1, with Kd values of 9.23×10^{-5} M and 2.03×10^{-6} M obtained for K8L9 and melittin, respectively (Figure 3C; Table S2). On the other hand, the increment of sensorgrams between magainin 2 and NRP1 was also found in the concentration-dependent manner of the peptides, but the Kd value for the interaction was 3.30×10^{-4} M, which was approximate 100-fold higher than that of melittin (Figure 3C; Table S2). Judging from the shape of curve after stopping the injection, dissociation of K8L9 was notably faster

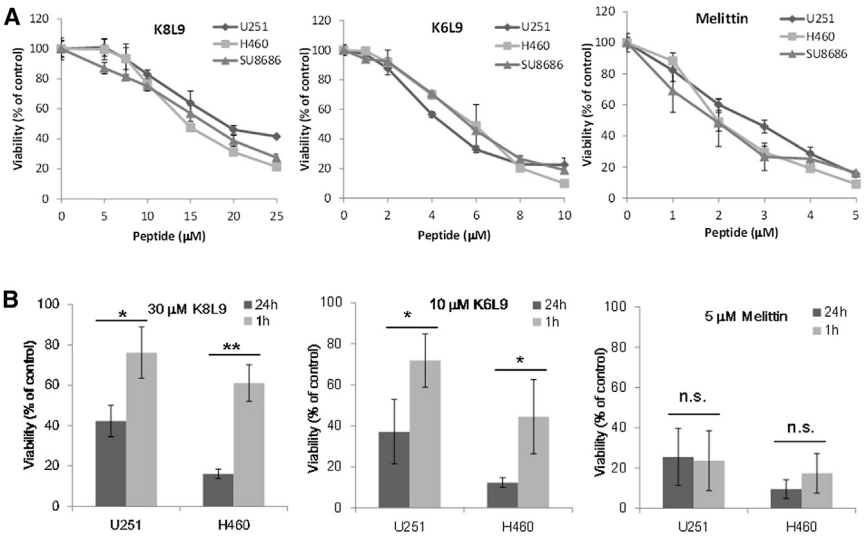

Figure 2. K8L9 Is Less Cytotoxic than K6L9 and Melittin

(A) U251, H460, and SU8686 cancer cell lines were cultured with various concentrations of K8L9 (left panel), K6L9 (middle panel), or melittin (right panel) for 24 hr, and cell viability was assessed using WST-8 reagent. The results are presented as the mean ± SD (bars) of triplicate determinations and were confirmed from the repeated experiments three or four times (Table S1).

(B) U251 and H460 were treated with 30 μM K8L9 (left panel), 10 μM K6L9 (middle panel), or 5 μM melittin (right panel) for 1 hr (gray columns) or 24 hr (black columns) before the peptide-containing medium was replaced with fresh medium for a further 24 hr culture. Cell viability was analyzed using WST-8. The results are represented as the means ± SD (bars) of three or four independent experiments. *$p < 0.05$, **$p < 0.01$. n.s., not significant.

than that of melittin, and we believe this to explain why no coprecipitation of NRP1 was observed with K8L9-biotin (Figure 3B). The binding affinity of magainin 2 to NRP1 was notably lower than that of K8L9 and melittin; also, the cytotoxic activity of magainin 2 was also weaker than K8L9 and melittin and only weakly attenuated by endocytosis inhibitors (Figure S4 and Table S3).

We proceeded to assess whether K8L9 induces downregulation of the endocytic receptors NRP1 and TfR. Incubation of SU8686 or T47D cells with K8L9 downregulated NRP1 or TfR, respectively (Figures 3D and S5). These results suggest that K8L9 and melittin interact with NRP1 and TfR.

Participation of Receptors in Cellular Uptake and Cytotoxic Activity of K8L9 and Melittin

To determine the potential role of receptors in the cellular uptake of K8L9 and melittin, the effect of siRNAs on each receptor was examined. Four cancer cell lines were transiently transfected with a negative control siRNA (siNC) or siRNAs specific to LRP1, NRP1, or TfR. The efficiency of gene knockdown was determined by western blot analysis. Knockdown of each receptor typically resulted in decreased K8L9-FITC uptake but did not change melittin-FITC uptake (Figure 4A). To determine the potential role of the receptors in the cytotoxic activity of K8L9 and melittin, the effect of each siRNA on cell viability was examined. K8L9 cytotoxicity was attenuated by knockdown of each receptor in all four cancer cell lines whereas the cytotoxicity of melittin was only attenuated by knockdown of LRP1 and TfR in U251 and H460 cell lines, respectively (Figure 4B; Table S4). These results suggest that K8L9 and melittin interact with NRP1, LRP1, and TfR, enter the cytoplasm via endocytosis, and affect cell damage outside and inside the plasma membrane.

Hsc70 Is One of the Interaction Partners of Melittin and K8L9

We tried to identify novel interaction partners of K8L9 and melittin by coimmunoprecipitation and mass spectrometry. U251 and H460 cells were treated with K8L9-biotin or melittin-biotin, and cell lysates were prepared. Complexes that formed with K8L9-biotin or melittin-biotin were captured using NeutrAvidin resin. The associated proteins were separated by SDS-PAGE and visualized by silver staining (Figure S6). Bands were excised from the gel and subjected to trypsin digestion, and the resulting peptides were analyzed by mass spectrometry. All bands were divided into nine groups (A–I) and liquid chromatography-mass spectrometry (LC-MS/MS) analyzed nine groups as one sample mixed with four or five bands and revealed an interaction of K8L9 and melittin with actins, tubulins, vimentin, elongation factor 1-α, methylcrotonoyl-CoA carboxylase β chain, and 40S/60S ribosomal proteins (Table S5). From these results, we focused on a protein chaperone, heat shock cognate 70 kDa (Hsc70) (Figure 5A). Since it is known that Hsc70 participates in endocytosis, chaperon-mediated autophagy, and virus entry, we hypothesized that Hsc70 may represent a novel and interesting interaction partner of melittin or K8L9. The interaction between Hsc70 and these peptides was further investigated by coimmunoprecipitation using NeutrAvidin resins and western blotting for Hsc70. Clear interaction bands for Hsc70 were detected in the presence of melittin-biotin only; no band was observed in the presence or absence of K8L9-biotin in any of the four cancer cell lines tested (Figure 5B). To compare the binding affinities of K8L9, melittin, and magainin 2 for Hsc70, binding to immobilized-Hsc70 on the surface of sensor chips was monitored using a Biacore analyzer. Increasing concentrations of K8L9 and melittin clearly increased Hsc70 binding, with Kd values of 6.02×10^{-5} M and 1.26×10^{-6} M obtained for K8L9 and melittin, respectively (Figure 5C and Table S2). It was also found that the binding affinity of Magainin 2 (2.42×10^{-4} M) to Hsc70 was lower than that of K8L9 and melittin (Figure 5C; Table S2).

Participation of Hsc70 in Cellular Uptake and Cytotoxic Activity of K8L9 and Melittin

To determine the potential role of Hsc70 in the cellular uptake of K8L9 and melittin, the effect of siRNA-mediated knockdown of Hsc70 was examined. Four cancer cell lines were transiently transfected with either Hsc70-specific siRNA or siNC. In all the

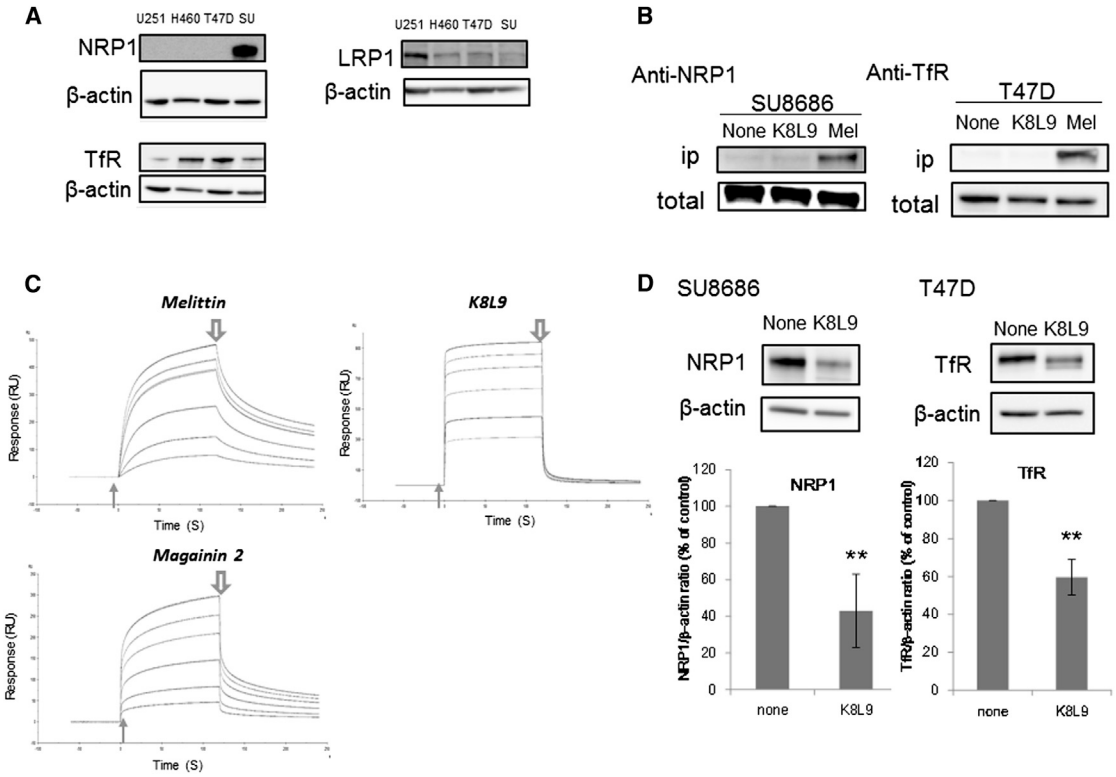

Figure 3. Interaction of K8L9 and Melittin with Endocytosis Receptors

(A) Analysis of NRP1, TfR, and LRP1 expression in cancer cell lines. Cell extracts from the indicated cancer cell lines were examined for NRP1, TfR, and LRP1 expression by western blot analysis using corresponding antibodies. β actin was used as a loading control. Bands were visualized by chemiluminescence.

(B) Immunoprecipitation of receptors associated with a biotinylated K8L9 or melittin. Whole-cell lysates were prepared from cancer cells incubated with K8L9-biotin or melittin-biotin and NeutrAvidin agarose resins were used to capture the proteins associated with the biotinylated peptides. Receptors were subsequently detected by western blotting.

(C) Sensorgrams of melittin, K8L9, or magainin 2 bound to immobilized NRP1, as determined using a Biacore biosensor. All peptides, melittin (1.25, 2.5, 5, 8, 10, and 12.5 μM), K8L9 (12.5, 25, 50, 80, 100, and 125 μM), or magainin 2 (12.5, 25, 50, 80, 100, and 125 μM) were injected over immobilized-NRP1, and the progress of binding was monitored by following the increase in the signal (response) induced by the analytes. Each analyte was injected in triplicate (n = 3), and three independent assays were performed for determination of dissociation constant (K_D) values. The thin and thick arrows indicate the initiation and termination of injection, respectively. Response was measured in resonance units (RUs).

(D) Western blot showing that K8L9 induced downregulation of NRP1 and TfR (upper panel, a typical example). The blots were stripped and incubated with an antibody against β actin, and the NRP1 or TfR bands were normalized to β actin levels using Image-J software and plotted on a bar graph as the means ± SD of three or four independent experiments (lower panel). **$p < 0.01$.

cancer cell lines, Hsc70 knockdown decreased K8L9-FITC uptake but did not change melittin-FITC uptake (Figure 6A). To determine the potential role of Hsc70 in the cytotoxic activity of K8L9 and melittin, the effect of Hsc70 siRNA on cell viability was examined. The cytotoxic activity of K8L9 and melittin was typically attenuated by Hsc70 knockdown in all four cancer cell lines (Figure 6B; Table S6). These results suggest that K8L9 and melittin interact with Hsc70 and that Hsc70 could participate in cell entry and cytotoxic activity of K8L9 and melittin.

K8L9 Alters Lysosomal Compartments

It is known that Hsc70 participates in chaperone-mediated autophagy by binding to cytosolic proteins and transporting them to the lysosome (Stricher et al., 2013). We tried to detect the autophagic phenomena stimulated by intracellular K8L9. The cytotoxic activity of K8L9 was attenuated by the autophagic inhibitors chloroquine and E64d (Figure S7A). To monitor the behavior of the compartments that are sequentially formed in autophagic flux, we first used the autophagic fluorescent probe, monodansylcadaverine (MDC), which can be quantitated by flow cytometry. A concentration-dependent increase in MDC fluorescence intensity was observed in U251 cells treated with K8L9 (Figure S7B). However, no enhancement of p62 expression, functional markers for autophagy, was observed in U251 and H460 cells treated with K8L9 (Figure S7C). Since the cytotoxic activity of K8L9 was attenuated by the cathepsin B inhibitor E64d, we hypothesized that K8L9 may alter the characteristics of lysosomal compartments.

We next analyzed the effect of K8L9 and melittin exposure on total cellular and cytosolic cathepsin B activity. K8L9 decreased the total cellular cathepsin B activity but increased cytosolic release of cathepsin B (Figure 7A, upper and lower panels, respectively). Melittin exposure weakly increased cytosolic release of cathepsin B (Figure 7A, lower panel). To monitor the lysosomal compartments, we used the lysosomotropic dye Lysotracker Green (LTG) and pH-probe Lysosensor blue, which

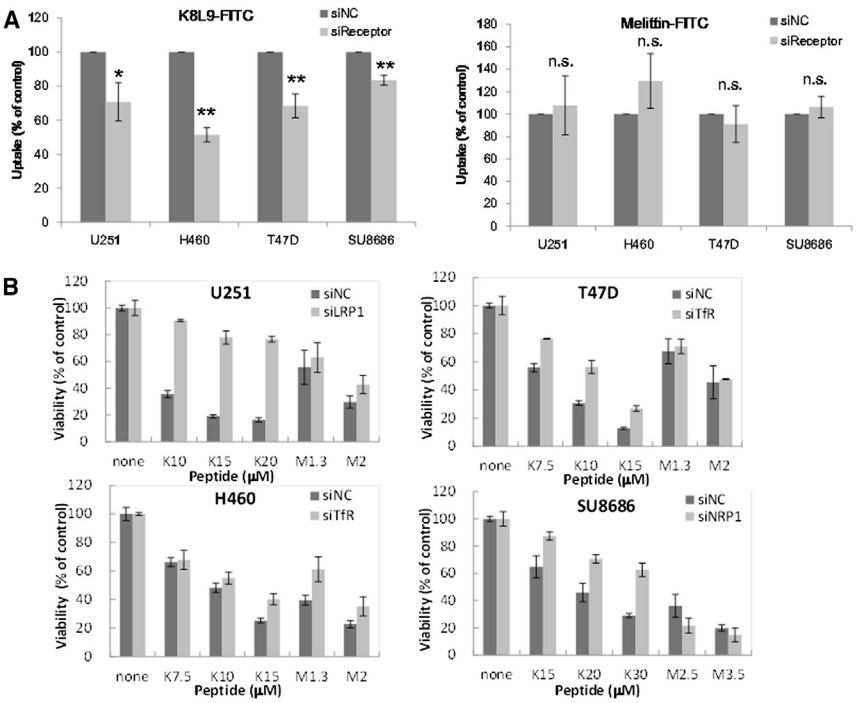

Figure 4. Participation of Receptors in Cellular Uptake of K8L9 and Melittin

(A) Effect of receptor siRNAs on K8L9-FITC and melittin-FITC cellular uptake. Cells were transfected with siRNAs specifically targeted against LRP1 (U251), TfR (H460 and T47D), or NRP1 (SU8686) for 72 hr prior to treatment with 3 μM K8L9-FITC (left panel) or 1 μM melittin-FITC (right panel) for an additional 1 hr. Uptake was quantitated as MFI. The results are represented as the means ± SD (bars) of three independent experiments. *$p < 0.05$, **$p < 0.01$. NC, negative control; n.s., not significant.

(B) Effect of receptor-siRNA on K8L9 cytotoxicity. Cells were transfected with siRNA specifically targeted against TfR, LRP1, or NRP1 for 72 hr prior to treatment with vehicle, K8L9 (K) or melittin (M) at the indicated concentrations for an additional 24 hr. The results are presented as the mean ± SD (bars) of triplicate determinations and were confirmed from the repeated experiments three or four times (Table S4).

can be quantitated by flow cytometry (Funk and Krise, 2012). A concentration-dependent increase in LTG or Lysosensor fluorescence intensity was observed in U251 cells treated with K8L9 and melittin (Figures 7B and S7D), and K8L9-TAMRA partially colocalized with the lysosome marker LTG (Figure 7C). Finally, we assessed the participation of Hsc70 on the effects of K8L9 on lysosomal compartments. Hsc70-siRNA inhibited the K8L9-stimulated increase in cytosolic release of cathepsin B and lysosomal compartments in U251 cells (Figure 7D). These data suggest that intracellular K8L9 alters lysosomal compartments.

DISCUSSION

We demonstrated that K8L9 and melittin are taken up into the cells via endocytic receptors and can subsequently affect lysosomal compartments. MAPs are classified into main three families, AMPs, CPPs, and amyloid β peptide, according to their initial apparent activity. They all share certain characteristics—namely, they are short (<50 amino acids), amphipathic, readily soluble in both aqueous and membrane environments and contain cationic sequences to mediate their interaction with negatively charged membrane components (Last et al., 2013).

CPPs are widely used as a powerful tool for a noninvasive cellular delivery of nucleic acids, peptides/proteins, and nanoparticles. Three representative CPPs, HIV-1 transactivator of transcription (Tat) protein-derived Tat peptide, antennapedia *Drosophila* homeodomain-derived Antp peptide, and artificial oligoarginine peptide have been extensively investigated and utilized for drug delivery, diagnostics, gene therapy, and various research applications. CPPs include many important sequences in cell entry and cellular traffic of virus such as TAT and antp. It is considered that there are many similarities between CPPs and virus in cell entry, cellular traffic, and endosomal escape, etc. (Varkouhi et al., 2011; Plank et al., 1998; Futaki et al., 2003). CPPs are known to enter cells by two principal mechanisms: direct cell membrane penetration and endosomal pathways. It has been reported that CPPs are internalized into the cytoplasm by several endosomal pathways, namely macropinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis (Räägel et al., 2010). Most CPPs are positively charged and are known to interact with negatively charged heparin sulfate proteoglycans on the cell membrane. Through this interaction, CPPs can effectively stimulate endocytosis via clustering glycosaminoglycans (GAGs), cell signaling, and actin rearrangements (Nakase et al., 2007). In addition to triggering endocytosis through GAGs, CPPs can also interact with cell surface receptors to stimulate cellular entry. It is reported that oligoarginine peptides can interact with CXCR4 or the laminin receptor to enter the cell via macropinocytosis (Tanaka et al., 2012; Zhou et al., 2012). Ezzat et al. (2012) demonstrated that PepFect14, a peptide nanocomplex with oligonucleotides, can enter cells via scavenger receptors, while Kondo et al., (2012) discovered tumor lineage-homing CPPs and demonstrated that CPP44 entered myelogenous leukemia cells via the M160 scavenger receptor family. Furthermore, TAT, antp, and oligoarginine are reported to induce internalization of the tumor necrosis factor (TNF) receptor (Fotin-Mleczek et al., 2005).

Amyloid β peptides are known to interact with various cell surface receptors, in particular, LRP1, RAGE, α7 nicotinic acetylcholine receptor, N-methyl-D-aspartic acid (NMDA) receptor, methanotrophic glutamate receptor, and p75 neurotrophin receptor (Takuma et al., 2009; Deane et al., 2004; Snyder et al., 2005; Renner et al., 2010; Nagele et al., 2002; Yamamoto et al., 2007). As mentioned above, three families of MAPs share similarities in how they attach and disintegrate the cell membrane and how they enter the cytoplasm. Furthermore, for some MAPs, receptor-mediated internalization is not restricted to a single receptor.

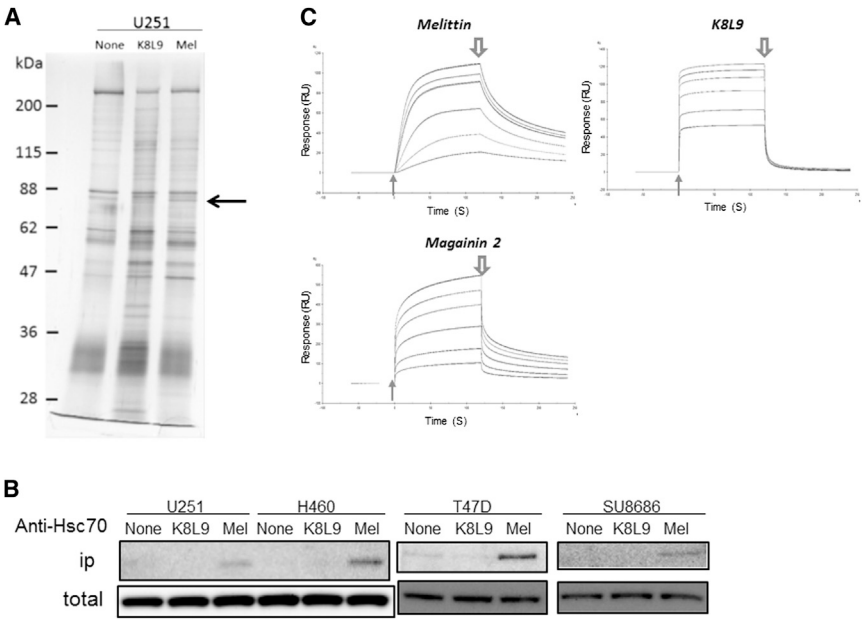

Figure 5. Hsc70 Is One of the Interaction Partners of Melittin and K8L9

(A) Immunoprecipitation of proteins associated with a biotinylated K8L9 or melittin. Whole-cell lysates were prepared from U251 cells treated with K8L9-biotin or melittin-biotin. Associated proteins were immunoprecipitated with NeutrAvidin and separated by SDS-PAGE prior to visualization by silver staining.

(B) Immunoprecipitation of Hsc70 with biotinylated K8L9 or melittin. Whole-cell lysates were prepared from cancer cells treated with K8L9-biotin or melittin-biotin, and associated proteins were captured using NeutrAvidin agarose resins. Hsc70 was detected by Western blotting.

(C) Sensorgrams of melittin, K8L9, or magainin 2 bound to immobilized Hsc70, as determined using a Biacore biosensor. All peptides, melittin (1.25, 2.5, 5, 8, 10, and 12.5 μM), K8L9 (12.5, 25, 50, 80, 100, 125 μM), or magainin 2 (12.5, 25, 50, 80, 100, and 125 μM) were injected over immobilized Hsc70 and, the progress of binding was monitored by following the increase in the signal (response) induced by the analytes. Each analyte was injected in triplicate (n = 3), and three independent assays were performed for determination of dissociation constant (K_D) values. The thin and thick arrows indicate the initiation and termination of injection, respectively. Response was measured in resonance units (RUs).

When considering the mechanism of receptor internalization by MAPs, it is interesting to consider that epidermal growth factor receptor (EGFR) can be ligand-independently activated and internalized by different stress factors including γ radiation, ultraviolet, oxidants, lipid rafts disruption, heat shock, and Hsp70 (Evdonin et al., 2006; Prenzel et al., 2001; Lambert et al., 2008). Based on this information, we hypothesize that K8L9 and melittin stimulate receptor internalization by perturbation of the cell membrane structure, including lipid rafts, triggering activation, and internalization of some cell membrane receptors, including some endocytic receptors, depending on the specific profile of receptor expression in a given cell line. Further investigation is required to test this hypothesis.

It is known that some cationic amphiphilic peptides and AMPs facilitate endosomal escape for cytosolic delivery, and modifications of these peptides have been reported to be useful for cytosolic delivery (Varkouhi et al., 2011). For example, although K6L9 and melittin have strong membrane-lytic and cytotoxic activities, stearylated versions of these peptides exhibit higher transfection efficiency and lower cytotoxicity (Zhang et al., 2013a, 2013b). Stearylation enables enhanced compaction of nucleic acids, facilitates endosomal escape, and reduces cytotoxic activity (Zhang et al., 2013a). K8L9 exhibited moderated membrane-lytic and cytotoxic properties compared with K6L9 (Figures 2A and 2B) and is thought to facilitate endosomal escape similar to the above peptides. K8L9 stimulated the cytosolic release of cathepsin B from lysosomes in the cells treated with the peptide (Figure 7A), and it is possible that intracellular trafficking of K8L9 is similar to that of the stearylated peptides mentioned above. Inhibition of cell entry of K8L9 by endocytosis inhibitors or siRNA of endocytosis-receptors decreased the cytotoxic activity of K8L9. It is considered that intracellular K8L9 could induce the damage of cell function against U251 cells.

In U251 cells treated with K8L9, increased lysosomal volume using LTG and vacuolization of the cytosol was observed (Figure 7B; data not shown). This phenomenon is known to be induced by long-term exposure to low concentrations of lysosomotropic compounds (Funk and Krise, 2012; Aki et al., 2012). In neutral pH conditions, these compounds are uncharged and can easily penetrate membranes, including those of lysosomes, but in the acidic environment of lysosomes, they become charged by protonation, cannot cross the lysosomal membrane, and become trapped in the lysosomes. Lysosomal compartments accumulating lysosomotropic compounds become enlarged and vacuolated by the influx of water (Agostinelli and Seiler, 2007). Lysosomotropic compounds have a lipophilic moiety and a basic moiety, similar to MLPs. Intraendosomal K8L9 is also presumed to become charged by protonation in low pH environments, leading to water influx and enlargement, which facilitates leakage or rupture of the lysosomal membrane.

We showed that Hsc70, identified as a melittin-interactive molecule, affected the cellular uptake and cytotoxic activity of melittin and K8L9. Heat shock cognate protein 70, Hsc70, belongs to the heat shock protein 70 (Hsp70) family, although Hsc70 is a constitutively expressed chaperone, whereas Hsp70 is inducible. Hsc70 and Hsp70 show some similarity in structure and function. Hsc70 contains two functional domains: the N-terminal ATPase domain and the C-terminal substrate binding domain. Hsc70 resides in the cytosol, nucleus, and close to membranes; prevents the aggregation of denatured, misfolded, or newly synthesized proteins; and takes part in cellular processes such as chaperone mediated autophagy and uncoating of clathrin-coated endosomes (Liu et al., 2012). It is reported that both Hsp70 and Hsc70 are released into the extracellular space, reside on the cell surface, and are required

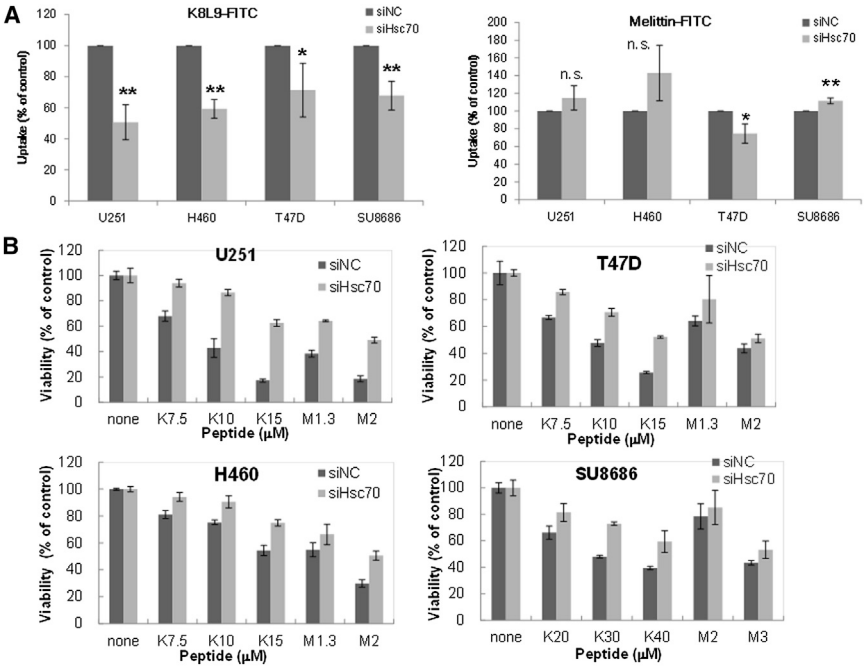

Figure 6. Participation of Hsc70 in Cellular Uptake of K8L9 and Melittin

(A) Effect of Hsc70-siRNA on cellular uptake of K8L9-FITC and melittin-FITC peptides. Cells were transfected with Hsc70-siRNA for 72 hr prior to treatment with 3 μM K8L9-FITC (left panel) or 1 μM melittin-FITC (right panel) for an additional 1 hr. Uptake was quantitated as MFI. The results are represented as the means ± SD (bars) of three or four independent experiments. *$p < 0.05$, **$p < 0.01$. NC, negative control; n.s., not significant.

(B) Effect of Hsc70-siRNA on K8L9 cytotoxicity. Cells were transfected with Hsc70-siRNA for 72 hr prior to treatment with vehicle, K8L9 (K), or melittin (M) at the indicated concentrations for an additional 24 hr. The results are presented as the mean ± SD (bars) of triplicate determinations, and were confirmed from the repeated experiments three to six times (Table S6).

to respond to damage and infection of the extracellular environment (Schmitt et al., 2007; Mambula et al., 2007; Novoselova et al., 2005). Some viruses, including rotavirus, reovirus, and hepatitis B virus, require Hsc70 for entry and infection of host cells (Pérez-Vargas et al., 2006; Ivanovic et al., 2007; Wang et al., 2010). Histatins 3 and 5, AMPs, and telomerase-derived CPP mixed with nucleic acids also enter cells via Hsp70 family members (Sun et al., 2008; Li et al., 2003; Lee et al., 2013). In neurological disease, Hsp70 family proteins attenuate aggregation and cytotoxicity associated with α synuclein, amyloid β, and polyglutamine in the intracellular and extracellular environment (Novoselova et al., 2005; Evans et al., 2006; Pemberton et al., 2011). Candidate molecules that are reported to assist in the transport of HSPs across the membrane include scavenger receptors, toll-like receptors, ABC transporters, flipping/flopping, and LRP1 (Basu et al., 2001; Thériault et al., 2006; Mambula and Calderwood, 2006). Quality control of proteins by HSPs has been investigated both intracellularly and within the membrane environment. De Maio (2011) proposed that the stress observation system performed by HSPs could extend to the extracellular, as well as intracellular, environment. According to this view, Hsc70 would participate in the cellular entry of some MAPs, including K8L9 and melittin, similar to viral infection.

It was previously reported that cationic L-CPPs were taken up more efficiently than their D counterparts in MC57 fibrosarcoma and HeLa cells (Verdurmen et al., 2011). On the other hand, L-K8L9 containing all L amino acids showed stronger cytotoxic activity and weaker sensitivity for endocytosis inhibitors than K8L9 containing five D amino acids (Figure S1). Many AMPs could have a potential as CPP by some suitable improvements such as introducing stearyl moiety (stearylated K6L9) and arranging D amino acids (K8L9). In this case, it is considered that suitable balance in membrane-lytic activity, cell entry, cytotoxicity, and endosomal escape is important. In this study, we investigated the cell entry via receptor endocytosis and cellular traffic of K8L9 and melittin. These sorts of studies in the cell entry and cellular traffic of MLPs and comparison between MLPs and CPPs may be useful for creating more excellent CPPs based on MLP/AMPs and promoting investigations of the cellular entry mechanisms of various exogenous molecules.

SIGNIFICANCE

We recently showed that the MLP K8L9 enters the cytoplasm via endocytosis-dependent and -independent pathways (Ohara et al., 2013). We hypothesized that some MLPs interact with extracellular receptors and enter the cell via endocytosis in a similar manner to amyloid β and CPPs. In this study, we demonstrated that K8L9 and melittin interact with receptors and enter the cytoplasm via endocytosis, tried to identify the molecules with which K8L9 and melittin interact, and analyzed the intracellular trafficking of K8L9 and melittin.

K8L9 and melittin interacted with the highly expressed endocytosis-receptors, NRP1, LDL-related protein receptor 1, and TfR. Silencing of these receptors by siRNAs attenuated the cytotoxic activity of K8L9 in four cancer cell lines. Intracellular K8L9 and melittin-triggered enlargement of the lysosomal compartments and cytosolic translocation of cathepsin B. Coimmunoprecipitation and mass spectrometry identified Hsc70 as a molecule that interacts with melittin. Knockdown of Hsc70 by siRNA attenuated the cellular uptake and cytotoxic activity of K8L9 and melittin. These findings suggest that K8L9 and melittin can enter cancer cells via receptor endocytosis following subcytotoxic exposure and subsequently affect the lysosomal compartments.

Many AMPs, including K8L9, could have a potential as CPP by some improvements such as stearylated K6L9. In this case, it is considered that suitable balance in membrane-lytic activity, cell entry, cytotoxicity, and endosomal escape is important. In this study, we investigated the cell

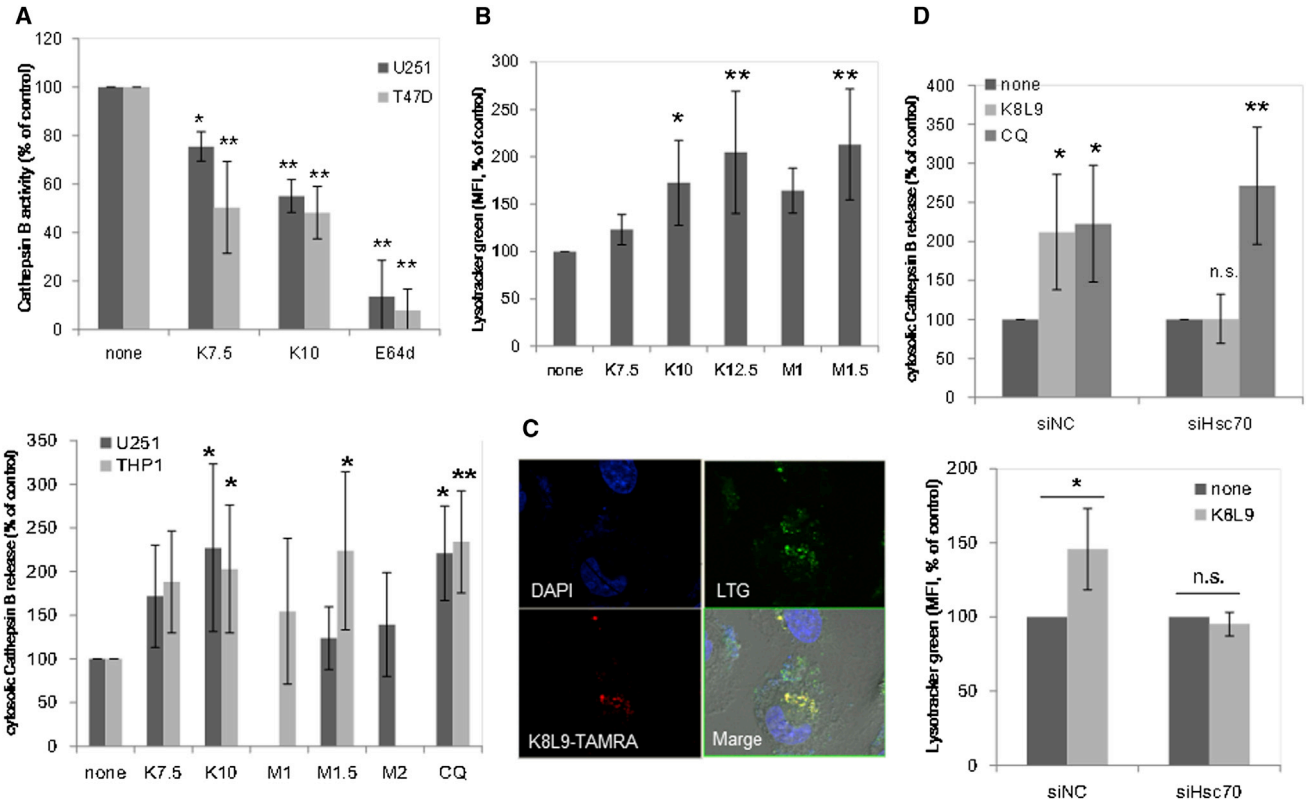

Figure 7. K8L9 and Melittin Alter Lysosomal Compartments

(A) The total cellular activity (upper panel) and the cytosolic release (lower panel) of cathepsin B were measured using z-RR-MCA, a fluorogenic probe, after 24 hr of drug treatment. The results are represented as the means ± SD (bars) of three to seven independent experiments. *$p < 0.05$, **$p < 0.01$.

(B) The volume of acidic compartments in U251 cells was analyzed after treatment with K8L9 (K) or melittin (M) for 24 hr by LTG staining and flow cytometry. The results are represented as the means ± SD (bars) of five independent experiments. *$p < 0.05$, **$p < 0.01$.

(C) Fluorescence signals from the K8L9-TAMRA peptide (red) and lysosome marker LTG distributed in the cytoplasm of U251 cells. Some K8L9-TAMRA are colocalized with LTG.

(D) Effect of Hsc70-siRNA on lysosomal compartments in U251 cells treated with K8L9. Cytosolic cathepsin B activity was measured using z-RR-MCA, a fluorogenic probe, after 24 hr of K8L9 or chloroquine (CQ) treatment (upper panel). The volume of acidic compartments was analyzed after treatment with 10 μM K8L9 for 24 hr by LTG and flow cytometry (lower panel). The results are represented as the means ± SD (bars) of three to five independent experiments. *$p < 0.05$. NC, negative control; n.s., not significant.

entry via receptor-endocytosis and cellular traffic of K8L9 and melittin. These sort of studies in the cell entry and cellular traffic of MLPs and comparison between MLPs and CPPs may be useful for creating more excellent CPPs based on MLP/AMPs and promoting investigations of the cellular entry mechanisms of various exogenous molecules.

EXPERIMENTAL PROCEDURES

Peptides

The following peptides were purchased from Invitrogen or American Peptide Company (bold and italic letters are D amino acids):

(1) K8L9: KL***L***LK***L***L***KK***LLK***L***LKKK-OH
(2) K8L9-FITC: KL***L***LK***L***L***KK***LLK***L***LKKK-FITC
(3) K8L9-TAMRA: KL***L***LK***L***L***KK***LLK***L***LKKK-TAMRA
(4) Melittin: GIGAVLKVLTTGLPALISWIKRKRQQ-OH
(5) Melittin-FITC: GIGAVLKVLTTGLPALISWIKRKRQQ-FITC
(6) K6L9: LK***L***LK***K***L***L***K***K***LL-NH_2
(7) Magainin 2: GIGKFLHSAK KFGKAFVGEIMNS-OH.

Endocytosis Inhibition Assay

Cells were pretreated with endocytosis inhibitors (0.75–1.00 μM cytochalasin D or 10 μM chlorpromazine) for 1 hr at 37°C in 96-well plates. The cells were incubated with different concentrations of peptides in 100 μl of medium for 24 hr at 37°C. Cell viability was then determined using WST-8 solution (Cell Count Reagent SF; Nacalai Tesque).

Cellular Uptake Analysis

Cells pretreated with endocytosis inhibitors were incubated with 3 μM K8L9-FITC or 1 μM melittin-FITC for 1 hr at 37°C. They were then collected and rinsed in 0.1 M sodium acetate buffer with 0.05 M NaCl (pH 5.5) to remove surface-bound FITC-labeled peptides (Mercer et al., 2010; Ohara et al., 2013) and analyzed using a LSR Fortessa cell analyzer (BD Biosciences).

Western Blotting

Western blot analyses were carried out as described previously (Kawamoto et al., 2013; Honjo et al., 2014). Briefly, protein extracts were prepared from cells lysed with lysis buffer (Promega), separated by SDS-PAGE, and transferred to nitrocellulose filters using the iBlot system (Invitrogen) according to the manufacturer's protocol. Blocked membranes were probed with antibodies and analyzed using Chemi-Lumi One Super reagent (Nacalai Tesque) and a LAS-3000 LuminoImage analyzer (Fujifilm).

Immunoprecipitation

After incubation for 1 hr in the presence or absence of 5 μM K8L9-biotin or 1 μM melittin-biotin, cells were washed twice with ice-cold PBS, scraped using a cell scraper, and collected by centrifugation at 300 *g* for 5 min. The pellet was lysed with M-PER (Mammalian Protein Extraction Reagent; Pierce) containing protease inhibitors. The protein content of cell lysates was determined using the Bio-Rad Protein Assay. Cell lysate containing 0.5–1.0 mg protein was incubated with 30–60 μl NeutrAvidin Agarose Resin solution (Pierce) at 4°C for overnight. The resin was collected by centrifugation, washed three times with Tris-buffered saline containing 0.03% Tween 20, and then boiled in SDS-PAGE sample buffer at 95°C for 5 min. The samples were separated by SDS-PAGE, and proteins were visualized using Sil-Best Stain One (Nacalai Tesque). Proteins were detected by immunoblotting, as described above.

Mass Spectrometry and Protein Identification

Products of gel trypsin digestion were suspended in 0.1% formic acid and subjected to LC-MS/MS analysis. For the analytical separation, a NanoLC-Ultra-2D Plus system with cHiPLC Nanoflex (Eksigent) was used in trap-and-elute mode, with a trap column (200 μm × 0.5 mm ChromXP C18-CL 3 μm 120 Å; Eksigent) and an analytical column (75 μm × 15 cm ChromXP C18-CL 3 μm 120 Å; Eksigent). Mobile phases used were 0.1% formic acid (loading solvent), 2% acetonitrile/0.1% formic acid (solvent A), and 80% acetonitrile/0.1% formic acid (solvent B). The gradient program for the separation was from 2% solvent B to 40% solvent B in 50 min. The separated samples were directly introduced into the TripleTOF 5600 system fitted with Nanospray III (AB SCIEX) for information-dependent data acquisition. Acquired data sets were analyzed by ProteinPilot software (AB SCIEX) using Paragon algorithm and a combined database of the UniProtKB/Swiss-Prot and known contaminants (AB SCIEX). The reliability of the protein identification was evaluated by the Unused ProtScore for each protein, which was calculated by the Pro Group algorithm (AB SCIEX).

Measurement of Cathepsin B Activity

To measure cytosolic enzyme activity, cells were treated with an extraction buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM pefabloc [pH 7.5]) containing 15 μg/ml digitonin for 12–15 min on ice. To measure the total cellular cathepsin B activity, cells were treated with the aforementioned extraction buffer containing 200 μg/ml digitonin for 12–15 min on ice. The cellular extracts were incubated with 50 μM of z-RR-AMC (7-amino-4-methylcoumarin; Peptide Institute) in cathepsin reaction buffer (50 mM sodium acetate, 4 mM EDTA, 8 mM DTT, 1 mM pefabloc [pH 6.0]) for 1 hr at 4°C (Nylandsted et al., 2004). Cathepsin B activity was measured by quantifying emission at 460 nm (excitation at 390 nm) using GloMax-Multi+ Detection System (Promega).

Localization of K8L9 in Living Cells

A total of 1 × 10^4 cells per well was seeded in eight-well chamber slides and incubated for 24 hr in RPMI 1640 medium containing 10% fetal bovine serum v/v. The cells were then incubated with 10 μM K8L9-TAMRA peptide in 200 μl of medium for 3 hr at 37°C. The cells were subsequently stained with LTG (Invitrogen) and Hoechst 33342 for 5 min and visualized using a FV1000 laser scanning confocal microscope (Olympus).

Lysosomal Volume and Permeability Assessment

To label lysosomal compartments, cells were incubated with 0.5 μM LTG for 5 min at 37°C and washed twice with PBS. Fluorescence integrity was evaluated using a LSR Fortessa cell analyzer.

Other Methods

For the remaining experimental procedures and a more detailed description of the above procedures, see Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.09.008.

AUTHOR CONTRIBUTIONS

M.K., T.H., K.O., and K.K. designed research. M.K., T.H., K.O., and S.I. performed research. M.K., T.H., K.O., and S.I. analyzed data, and M.K., T.H., S.I., and K.K. wrote the paper.

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