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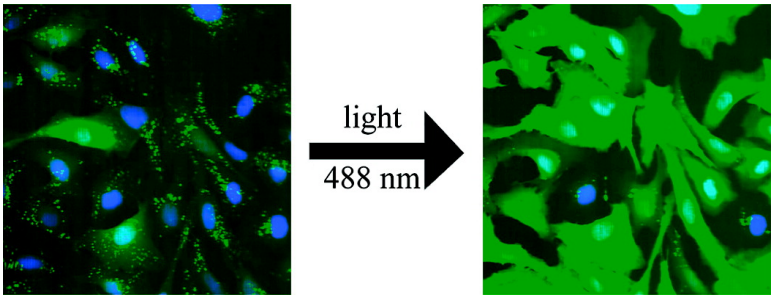

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Specific Redistribution of Cell-Penetrating Peptides from Endosomes to the Cytoplasm and Nucleus upon Laser Illumination

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Transduction of biologically active molecules across plasma membranes has been a persistent challenge. In the past decade, the realization that certain peptide sequences, termed cell-penetrating peptides (CPPs), have the ability to transport varied cargo molecules across cell membranes has provided a novel mechanism for overcoming the poor uptake of these biomolecules.[1−4] Recent confocal microscopy studies using live cells have revealed that, for many CPPs, cellular uptake is energy dependent and endocytotic in nature.[5−10] Trapping of CPPs in endosomal compartments can reduce the biological activity of their cargo due to the lack of cytoplasmatic distribution and/or their degradation in the late endosomes. Therefore, if the cargo molecules fused to the CPPs could be released from the vesicles into the cytoplasm early in the endosomal pathway, their ability to interact with their biological targets could be significantly increased. Here, we report that it is possible to use laser illumination to trigger the specific redistribution of a cargo peptide fused to a fluorophore labeled-CPP from endosomes to the cytoplasm and nucleus. This photoinduced redistribution of CPP-fused peptides could have general application in improving the CPP-mediated uptake of biomolecules into the cytoplasm and consequently their biological activity.

For these studies, we used a fluorescein-labeled CPP−cargo peptide conjugate, Fluorescein−RRRRRRRW−GREEEVQD (Fl−R_7W−VQD). This cargo sequence corresponds to residues in the C-terminus of the cystic fibrosis transmembrane regulator (CFTR), and was chosen because of its clear endocytotic uptake in U2OS cells when fused to the CPP sequence R_7W (Figure 1A). It has been shown that some photosensitizers that insert into the lysosomal membrane, such as tetra(4-sulfonatophenyl)porphine, can induce endosomal opening when irradiated by light.[11,12] The mechanism of this photochemical endosomal opening is not well understood, but it is thought to occur through local damaging of the membrane by short-lived reactive oxygen species generated by the light. Because the CPPs are thought to interact with the membranes,[13−15] we postulated that a similar effect could be observed when using CPP−cargo peptides labeled with a fluorophore. Indeed, it was found that Fl−R_7W−VQD could be quickly redistributed from the endosomal vesicles into the cytoplasm and nucleus of cells by raster scanning the cells with laser illumination (∼36 mW) at 488 nm while adjusting the focal plane (Figure 1B). The redistribution effect was observed using two different confocal microscopes, an InCell3000 (Figure 1) and a Nikon C1 (Figure S1). Z-scans revealed that the diffuse distribution of the peptide observed is even throughout the cytoplasm and nucleus (data not shown).

A recognized effect of photochemical reactions in cells is cytotoxicity. Under the laser illumination conditions used, the outer cell membrane remains intact following irradiation, as indicated by the lack of staining of the cells by propidium iodide after redistribution (data not shown). Moreover, the cells utilized in these

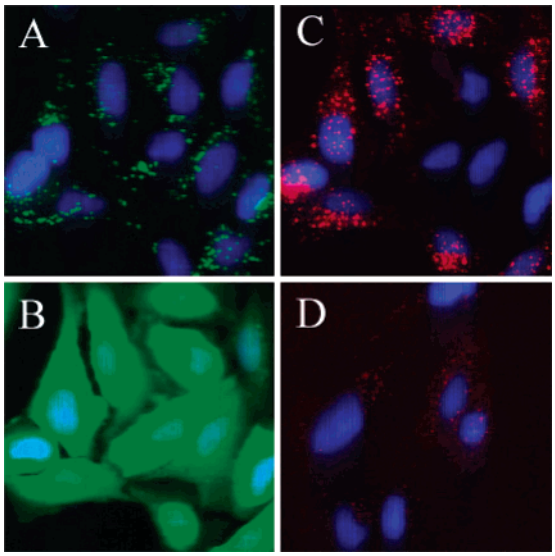

Figure 1. Redistribution of Fl−R_7W−VQD and viability of U2OS cells. U2OS cells were incubated with 20 μM Fl−R_7W−VQD peptide for 2 h at 37 °C, washed, and incubated for 3 h at 37 °C in peptide-free media. Redistribution was induced with laser irradiation at 488 nm and fluorescence measured in three channels using 346, 488, and 633 nm excitation. Cells were stained with Hoechst 33258 (blue), Fl−R_7W−VQD (green), and CypHer5E-anti-VSV-G (red). (A) Before redistribution. (B) After laser-induced redistribution. (C) After laser-induced redistribution, binding of CypHer5E-anti-VSV-G antibody, and stimulation with isoproterenol. (D) After laser-induced redistribution, binding of CypHer5E-anti-VSV-G, but without isoproterenol stimulation.

experiments overexpressed the β2-adrenergic receptor (β2-AR), with a vesicular stomatitis virus glycoprotein (VSV-G) peptide tag fused at the N-terminus. It was therefore possible to monitor whether these cells were still responsive after laser illumination by activating the receptor with the agonist isoproterenol and monitoring receptor internalization with a CypHer5E-labeled anti-VSV-G antibody. The CypHer5E fluorophore is pH sensitive, and its emission intensity increases at the acidic pH of endosomes.[16] Therefore, it is used as a marker for receptor internalization. Figure 1C shows that after laser illumination, the cells were responsive and β2-AR was internalized upon agonist stimulation. As a control, Figure 1D shows lack of receptor internalization in nonstimulated cells (also after laser illumination). Finally, under standard redistribution conditions, cells did not show a rounded morphology consistent with cytotoxicity for at least 4 h, after irradiation.

The same laser-triggered redistribution from endosomal vesicles to cytoplasm and nucleus was observed when the same peptide sequence was labeled with a different fluorophore, AlexaFluor633, Al−R_7W−VQD. The redistribution occurred when the cells were irradiated with a 633 nm laser source, with a light energy of ∼7

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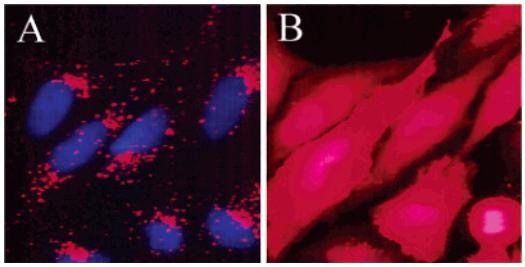

Figure 2. Redistribution of Al-R_7W−VQD with a 633 nm laser. U2OS cells were incubated for 2 h at 37 °C with 20 μM Al-R_7W−VQD (red), 10 μM R_7W−VQD, and 5 μM Hoechst 33258 (blue), washed, and incubated for 3 h at 37 °C with compound-free media. Redistribution was induced with laser illumination at 633 nm and fluorescence measured in two channels using 346 and 633 nm excitation. (A) Before irradiation. (B) After laser-induced redistribution.

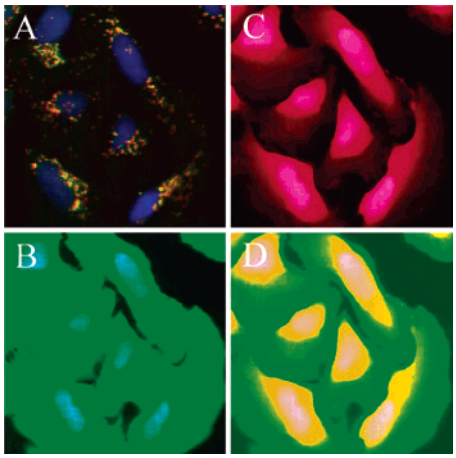

Figure 3. Colocalization of peptides before and after redistribution. U2OS cells were incubated with 20 μM Fl−R_7W−VQD (green), 5 μM Al−R_7−DTRL (red), and 5 μM Hoechst 33258 (blue) for 2 h at 37 °C, washed and incubated for 3 h at 37 °C in compound-free media. Redistribution was induced with laser illumination at 488 nm, and fluorescence measured in three channels using 346, 488, and 633 nm excitation. (A) Before redistribution (overlay of Hoechst, Fl−R_7W−VQD and Al−R_7−DTRL). After laser-induced redistribution, (B) overlay of Hoechst and Fl−R_7W−VQD; (C) overlay of Hoechst and Al−R_7−DTRL; and (D) overlay of Hoechst, Fl−R_7W−VQD and Al−R_7−DTRL.

mW (Figure 2A and B) (no redistribution occurred when cells were irradiated with a 488 nm laser). However, to obtain the same dramatic redistribution observed for the fluorescein-labeled peptide, it was necessary to increase the total amount of peptide loaded into the cells from 20 to 30 μM, by adding unlabeled peptide (Figure 2 A and B). This observation suggests the possibility that for each fluorophore a critical total peptide concentration (labeled and unlabeled) is needed in the vesicles, and that the fluorophore-labeled peptide can be used as a tracer to trigger the release of the biomolecules upon illumination.

The ability to use a fluorophore-labeled CPP-conjugated peptide to phototrigger the redistribution of another CPP-conjugated sequence was explored further by using mixtures of the two labeled peptides, Fl−R_7W−VQD and Alexa 633−RRRRRRR−GREEEVQDTRL (Al−R_7−DTRL). When added together to the cells, the two peptides co-localize in the endosomal vesicles (Figure 3A). Following laser irradiation at 488 nm, both the fluorescein-labeled peptide and the Alexa 633-labeled peptide were found to redistribute into the cytoplasm and nucleus (Figure 3). The same effect was also observed when the 633 nm laser was used to irradiate the cells (Figure S2). The observed redistribution of both peptides when only one of the fluorophores is excited suggests that the vesicles are disrupted, causing the release of their contents into the cytoplasm. This supports the idea that one fluorophore−CPP−peptide sequence could be used as a general phototrigger to redistribute other CPP−biomolecules of interest.

In conclusion, a novel method for delivering CPP-conjugated biomolecules into the cytoplasm and nucleus of cells based on laser illumination has been described. The photochemistry is mild enough that a biological response is observed after laser illumination, and under the proper conditions cells retain morphology for 24 h following laser irradiation. It appears that the phototreatment opens the endosomal compartments, releasing their contents into the cytoplasm and nucleus. We are now further exploring the broader applicability of using a fluorophore−CPP−peptide to photoinduce the redistribution of a variety of biomolecule cargos, both fused and non-fused to CPPs, to the cytoplasm.

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Supporting Information Available: Detailed experimental methods and supplemental microscope images. This material is available free of charge via the Internet at http://pubs.acs.org.

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