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Disulfide and thioether linked cytochrome *c*-oligoarginine conjugates in HeLa cells

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ABSTRACT

The intracellular processing and the apoptotic activity of conjugates of oligoarginine and cytochrome *c* (Cyt *c*) were studied. Disulfide and thioether linked conjugates were prepared by coupling Cyt *c* to cysteinyl-nonaarginine, $C(R)_9$, through SPDP and SMPB cross-linkers, respectively. Internalization of the radiolabeled conjugates was measured, and biological activity via induction of apoptosis was determined using the annexin V and the acridine orange assays in HeLa cells. The internalization of both conjugates is increased when compared to that of Cyt *c* alone. However, the biological activity of the internalized Cyt *c*, indicated by apoptosis in HeLa cells, was expressed only in the thioether (SMPB) conjugate, but not the disulfide (SPDP) conjugate or free Cyt *c*. The addition of the proteasomal inhibitor MG132 increased the apoptotic activity of both the disulfide conjugate and free Cyt *c*, but not the thioether conjugate. Our results suggest that the intracellular cleavage of the linker in cell penetrating peptide conjugates is critical in determining the fate and activity of biologically degradable cargo molecules.

1. Introduction

Peptides characterized as cell penetrating peptides (CPP) have recently attracted attention as a novel approach for the efficient intracellular delivery of oligopeptides, oligonucleotides and other bioactive macromolecules, mainly into the cytoplasm of mammalian cells (Murriel and Dowdy, 2006; Patel et al., 2007). CPPs are typically classified as cationic in nature, containing primarily arginine and lysine residues, and include the basic domain of the nuclear transcription activator Tat (47–57) (YGRKKRRQRRR) encoded by HIV-1, *Drosophila* Antennapedia, Antp (43–58) (RQIKIYFQNRRMKWKK), and small oligoarginine $(R)_n$ and oligolysine $(K)_n$ peptides (Fawell et al., 1994; Vives et al., 1997; Derossi et al., 1994). CPP-conjugates offer a unique and efficient drug transport mechanism directly into the cytosol of cells, termed membrane transduction, which can be used to deliver macromolecules and hydrophilic drugs with cytosolic target sites. Although it has been shown that CPPs are metabolically degraded (Foerg and Merkle, 2008), the intracellular processing of CPP peptides, as well as their macromolecular conjugates, has not been elucidated.

Cytochrome *c* is a hemoprotein with a molecular weight of 12 kDa and a highly conserved amino acid sequence across the spectrum of species, including plants, animals, and many unicellular organisms (Salemme, 1977). Cyt *c* is a nuclear encoded protein located in the inner membrane space of all mitochondria. The molecule typically functions in the electron transport system of oxidative phosphorylation to release energy in the form of ATP. When released from the mitochondria in response to pro-apoptotic stimuli, Cyt *c* plays a pivotal role in the regulation of apoptosis (Jiang and Wang, 2004). The apoptotic function of Cyt *c* makes it an intriguing peptide for cytoplasmic delivery. Current methodologies such as pinocytotic loading and microinjection of Cyt *c* have been successful in stimulating the apoptotic cascade, but under very harsh conditions to the cell (Gilmore et al., 2001; Li et al., 1997; Chang et al., 2000; Zhivotovsky et al., 1998).

In this report, we explored oligoarginine as a CPP-carrier to increase the cytosolic transport of Cyt *c* in cultured HeLa cells. We used both the disulfide and the thioether linkage to prepare the conjugates. With these two different conjugates, as well as the unconjugated Cyt *c*, the intracellular processing and apoptotic activity of transduced proteins in the cytoplasmic compartment was investigated.

2. Materials and methods

2.1. Cell culture

Human cervical carcinoma HeLa cells (ATCC, Manassas, VA) were grown in Eagle's minimum essential medium (EMEM)

Abbreviations: CPP, cell penetrating peptide; Cyt *c*, cytochrome *c*; SPDP, *N*-succinimidyl 3-(2-pyridyldithio) propionate; SMPB, succimidyl 2-[*p*-maleimidophenyl]butyrate.

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(GIBCO-BRL, Carlsbad, CA) containing 10% fetal bovine serum, non-essential amino acids and sodium pyruvate. The cells were incubated at 37 °C, 5% CO_2 and replenished with fresh medium the day before confluence, at which time the assays were performed.

2.2. Conjugate preparation and labeling

The disulfide bonded conjugate was prepared by combining equine Cyt *c* (Sigma, St. Louis, MO) with *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Pierce, Rockford, IL) at a 1:2 molar ratio at 4 °C for 30 min. The solution was dialyzed overnight and a recovery assay was performed to determine the concentration of Cyt *c* in the conjugate. Reduction of SPDP-modified Cyt *c* by dithiothreitol (DTT) was performed to determine the exact molar ratio of the conjugate by measuring the increase in absorption at 343 nm due to the release of thiopyridine. SPDP-modified Cyt *c* was combined with a 4-fold excess of $C(R)_9$ (synthesized by Genemed Biotech, San Antonio, TX) for 1 h at room temperature and purified by size exclusion chromatography using a Sephadex G-25 column. The thioether linked conjugate was prepared by combining Cyt *c* with sulfosuccinimidyl 4-[*p*-maleimidophenyl]butyrate (Sulfo-SMPB) (Pierce, Rockford, IL) in the same manner as the SPDP conjugate. After overnight dialysis and a recovery assay to determine the Cyt *c* concentration in the conjugate, the SMPB-modified Cyt *c* was combined with 4-fold excess of $C(R)_9$ for 30 min at room temperature and purified using a Sephadex G-25 column. The conjugates were assayed for amino acid analysis (Life Science Research Center, National Tsing Hua University, Hsinchu, Taiwan). The final conjugates consisted of between 0.5 and 2 moles of $C(R)_9$ in each mole of Cyt *c*, based on the number of arginyl and alanyl residues as 2 and 6, respectively, in equine Cyt *c*. Cyt *c* in its free form, and the Cyt *c* conjugates were labeled with $Na^{125}I$ (ICN, Irvine, CA) using the chloramine-T method (Sonoda and Schlamowitz, 1970).

2.3. Uptake assays

Confluent HeLa cell monolayers grown in 6-well culture plates (Corning, Acton, MA) were incubated in serum free medium (SFM) containing 5 μg/mL iodinated Cyt *c*-$C(R)_9$ conjugates or Cyt *c* for 1 h at 37 °C. The cell pellets were washed with phosphate buffered saline (PBS), isolated following treatment with trypsin-EDTA, washed with PBS, and dissolved in 1N NaOH. The amount of internalized conjugate was assayed using a Gamma counter (Packard, Downers Grove, IL), and the total cell protein content in each sample was determined by the BCA protein assay (Pierce).

2.4. Biological activity assays

To determine the cytotoxicity of the conjugates, HeLa cells were grown overnight to approximately 50% confluence in 24-well culture plates (Corning) and incubated in serum free medium containing 10, 20, 40 or 80 μg/mL Cyt *c*-$C(R)_9$ conjugate, 20, 40 or 80 μg/mL Cyt *c* or 5, 10 or 20 μg/mL $C(R)_9$, for 1 h at 37 °C. At 1 h, fetal bovine serum was added to 5% and the cells were incubated until cells in the control wells reached confluence. The cells were washed with PBS, dissolved in 1N NaOH and the total cell protein content was determined by the BCA protein assay (Pierce).

Apoptosis was assayed by using both the annexin V/propidium iodide (Sigma), and the acridine orange (AO) (Sigma) methods as previously described, with slight variation (van Engeland et al., 1998; Baskic et al., 2006). Briefly, HeLa cells were plated in a 12-well culture plate and allowed to attach overnight. Cells were changed to serum free medium and treated with 80 μg/mL Cyt *c*-$C(R)_9$ conjugates or Cyt *c*, 20 μg/mL $C(R)_9$ or 10 μg/mL cycloheximide (Sigma) for 1 h at 37 °C. At 1 h, fetal bovine serum was added to 5% and the cells were incubated for 3 more hours. Cells were washed with PBS, isolated following treatment with trypsin-EDTA, and washed with PBS. For the annexin V/propidium iodide assay, the cells were washed with PBS and resuspended in 1× binding buffer (provided with the annexin V assay kit) at a concentration of approximately 1×10^6 cells/mL. To 500 μL of each cell suspension, 5 μL of annexin V-FITC and 10 μL of propidium iodide was added. The cell suspension was then incubated at room temperature for 10 min while protected from light. The cells in the early stage of apoptosis (annexin V positive/propidium iodide negative) were determined using flow cytometry as these cells appeared in the lower right quarter of the scope (USC/Norris Comprehensive Cancer Center Flow Cytometry and Immune Monitoring Core). For AO assay, cells were washed with PBS, then stained with AO solution (4 μM) on ice for 5 min, washed again with PBS, and observed using a fluorescence microscope.

2.5. Degradation assays

Proteasome involvement in the intracellular processing of Cyt *c*, either as free protein or as the conjugate, was determined by performing cytoplasmic localization assays for subcellular fractionation and the annexin V/propidium iodide assays for apoptosis.

To measure the fraction of degradation product in subcellular fractionation, HeLa cells in T-75 flasks were incubated with ^{125}I-labeled Cyt *c* conjugates, with or without the treatment of the proteasome inhibitor, MG132, and processed as previously reported (Baskic et al., 2006). Briefly, confluent HeLa cell monolayers grown in T-75 flasks (Corning) were incubated in serum free medium containing 3 μg/mL of ^{125}I-labeled free or conjugated Cyt *c* with and without 10 μM of MG132. FITC-dextran (70 kDa) (FD) (Sigma) was also added as a marker for endocytotic vesicles. After treatment for 1 h at 37 °C, the monolayers were trypsinized, washed with 0.5 mg/mL heparin-PBS, and the cell pellets were homogenized by using a Balch cell press (H&Y Enterprises, Redwood City, CA) (Balch and Rothman, 1985). The cell homogenate was centrifuged and the postnuclear supernatant was fractionated using Sephacryl S-500 (Amersham, Piscataway, NJ) size exclusion chromatography (1 cm × 13 cm column dimensions). The amount of labeled protein localized in the vesicles (first peak) versus the cytosol (second peak) was calculated using the equations previously described (Zaro and Shen, 2003). The amount of labeled protein localized in the third peak, which represented peptide fragments smaller than the intact Cyt *c*, was considered the degradation product.

For apoptotic assay, HeLa cells were seeded in a 24-well culture plate and allowed to attach overnight. The cells were incubated

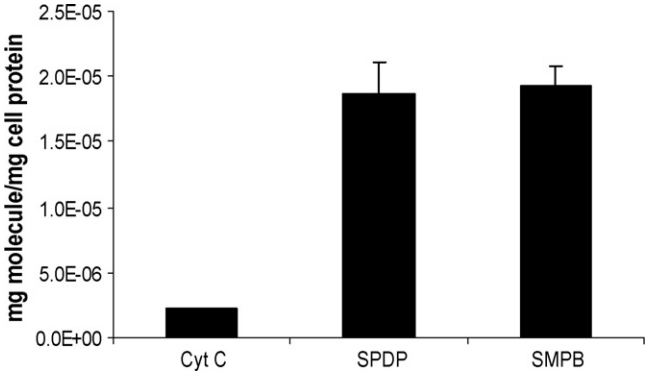

Fig. 1. Increased conjugate uptake. HeLa cultured cell monolayers were incubated with 5 μg/mL Cyt *c*-$C(R)_9$ conjugates or Cyt *c* alone for 1 h. Cells were washed, trypsinized, dissolved in NaOH and the amount of internalized peptide was assayed using a Gamma counter.

in serum free medium with either 0 or 10 μM MG132 proteasome inhibitor for 30 min at 37 °C. At 30 min, 80 μg/mL Cyt *c*-$C(R)_9$ conjugates or Cyt *c* alone was added and the cells incubated for 1 h at 37 °C. At 1 h, fetal bovine serum was added to 5%. After another 3 h incubation at 37 °C, cells were processed for cytoplasmic localization and annexin V/propidium iodide assays as described above.

2.6. Data analysis

For the quantitative analyses, the data is presented as average plus standard deviation with $n > 3$ for all experiments. The Student's *t*-test was utilized to compare data sets, where values with $p < 0.05$ were considered statistically significant, and values with $p > 0.1$ were considered insignificant.

3. Results

3.1. Enhanced uptake of conjugates

As expected, Cyt *c* exhibited very little uptake into HeLa cells. The conjugation with $C(R)_9$, either with a disulfide or a thioether linkage, increased the amount of Cyt *c* located in the cell by approximately 8-fold as shown in Fig. 1.

3.2. Retention of biological activity

To determine cytotoxicity, cultured HeLa cells were treated with increasing concentrations of either disulfide Cyt *c*-$(R)_9$ conjugate, thioether Cyt *c*-$(R)_9$ conjugates, unconjugated Cyt *c* or unconjugated $C(R)_9$. The results indicated that both conjugates induce cytotoxicity, but the thioether conjugate is more effective, eliciting approximately 30% cytotoxicity at a concentration of 80 μg/mL (Fig. 2).

To determine if cytotoxicity was due to apoptosis, cultured HeLa cells were assayed using the annexin V assay. Cells were treated with 80 μg/mL Cyt *c*-$(R)_9$ conjugates or Cyt *c*, 20 μg/mL $C(R)_9$ and various controls. Results show 26% apoptosis in the thioether conjugate, but no significant enhancement of apoptosis in any other condition compared to control cells of 12% apoptosis (Fig. 3). Acridine orange (AO) accompanied by laser scanning microscopic (LSM) pictures served as a visual confirmation of apoptosis. Cells were treated in the same manner as those in the annexin V assay. Comparison of the control and conjugates to a positive control of cycloheximide verified that apoptosis was only occurring in the non-reducible thioether conjugate (Fig. 4).

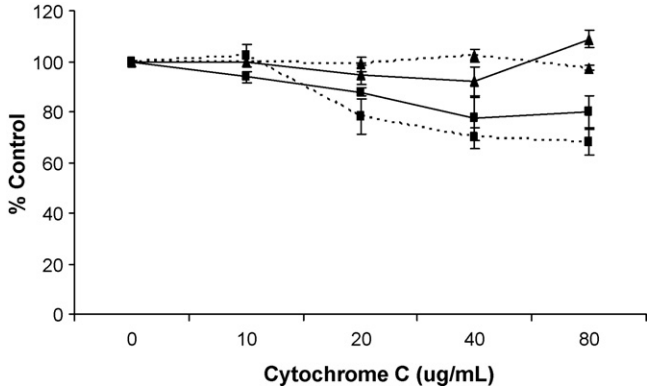

Fig. 2. Cytotoxicity of conjugates. HeLa cultured cell monolayers were incubated with increasing concentrations of Cyt *c*, conjugates or oligoarginine. Cells were washed, dissolved in NaOH and total cell protein determined. SPDP (solid line, square), SMBP (dashed line, square), Cyt *c* (solid line, triangle), $C(R)_9$ (dashed line, triangle). At 80 μg/mL: SPDP or SMPB vs. Control: $p < 0.05$; SPDP vs. SMPB: $p < 0.05$.

3.3. Cytoplasmic localization

The intracellular localization of the Cyt *c* and the Cyt *c* conjugates was evaluated by using a subcellular fractionation method as described previously (Zaro and Shen, 2003). In addition to the first peak of vesicular localization and the second peak of cytosolic localization, a third peak was also observed. This peak was assumed to be the degradation products of Cyt *c*, because the molecular weight of this peak was smaller than that of a Cyt *c* standard (Fig. 5). No such peak was detected when Cyt *c* was incubated with cell extracts from HeLa cells, suggesting that the degradation occurred only intracellularly in the living cells (data not shown).

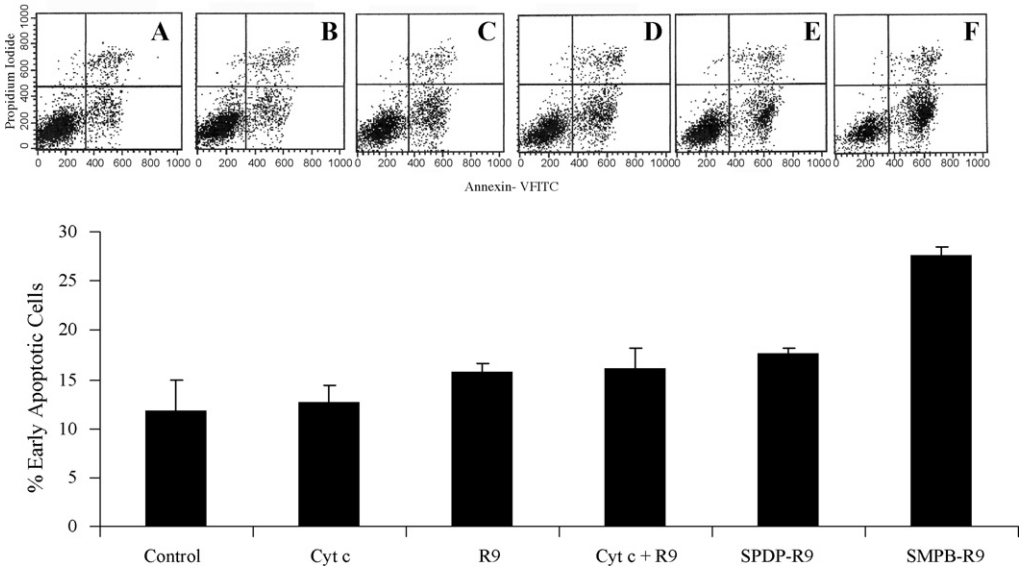

Fig. 3. Annexin V apoptotic effect. HeLa cultured cell monolayers were incubated with PBS (A), 80 μg/mL Cyt *c* (B) 20 μg/mL $C(R)_9$ (C), 80 μg/mL Cyt *c* + 20 μg/mL $C(R)_9$ unconjugated (D), 80 μg/mL Cyt *c* disulfide conjugate (E), or 80 μg/mL Cyt *c* thioether conjugate (F). Cells were washed, suspended in binding buffer and stained with both annexin V-FITC and propidium iodide. Representative scans are shown, the figure represents results obtained from $n = 3$, error bars represent the standard deviation.

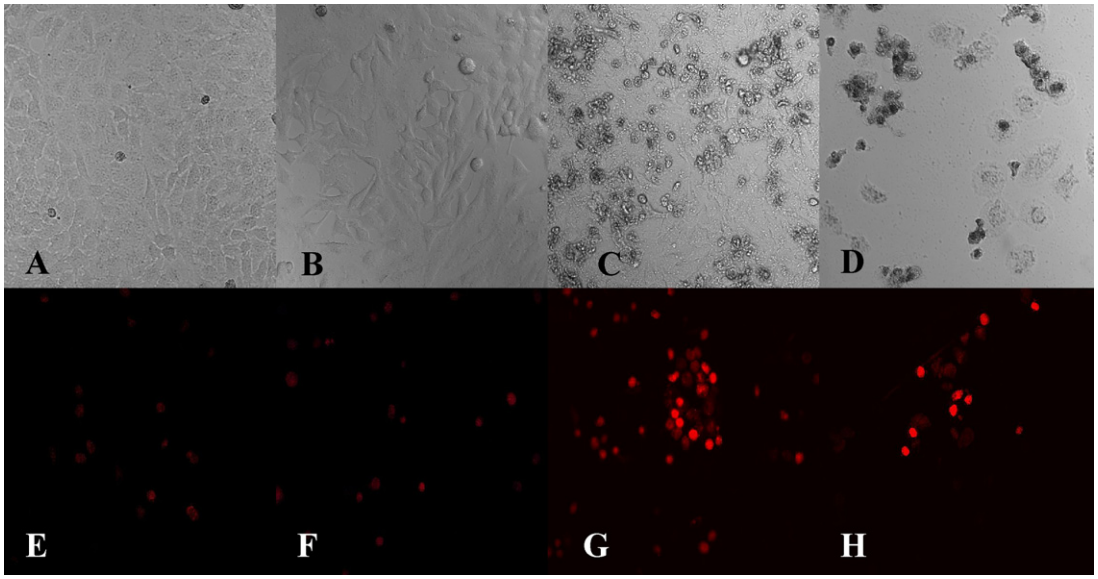

Fig. 4. Acridine orange apoptosis and laser scanning microscopy. Cyt *c* control (A and E) vs. SPDP conjugate (B and F) vs. SMPB conjugate (C and G) and cycloheximide (positive control) (D and H). Pictures before staining (A–D) and acridine orange staining (E–H). PBS and $C(R)_9$ results not shown, no cell death observed.

3.4. Proteasome inhibition

When coincubated with MG132, a proteasome inhibitor, Cyt *c* released from the disulfide linked conjugate, as well as unconjugated Cyt *c*, exhibited a significant increase of apoptotic activity (Fig. 6). On the other hand, MG132 had no significant effect on the thioether conjugate (Fig. 6). This result suggests that the degradation of Cyt *c* by proteasomes was responsible for the lack of apoptotic activity of cell internalized Cyt *c* and its disulfide conjugate. Furthermore, it suggests that the thioether conjugate can avoid the proteasomal degradation and can exert its apoptotic activity as the intact conjugate form.

To confirm the difference in intracellular processing of the disulfide and the thioether conjugates, subcellular fractionation of cell homogenate was performed (Zaro and Shen, 2003). An example elution profile of the disulfide conjugate with the third peak is shown in Fig. 5, compared to Cyt *c* elution without cellular incubation, suggesting that the third peak is a cellular degradation product. When cells were incubated in the presence of MG132 and assayed for internalization by subcellular fractionation, degradation is decreased and the amount localized in the cytosol is increased in disulfide conjugate-treated cells (Fig. 7A). However, no significant difference in the fraction of either cytosolic or degradation amounts was found in thioether conjugate (Fig. 7B), suggesting degradation of the intact conjugate through other non-proteasomal pathways.

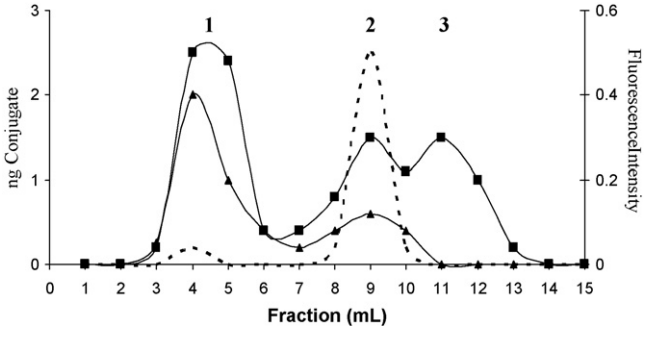

Fig. 5. Example elution profile of oligoarginine conjugate with the degradation peak. Postnuclear supernatant of HeLa cells that were incubated with iodinated Cyt *c*-$(R)_9$ disulfide conjugate and separated into the (1) vesicular, (2) cytosolic and (3) degradation peaks (squares) as described in previous report (Baskic et al., 2006). FITC-dextran elutes in the endocytic peak, with a second smaller leakage peak (triangles). The elution profile of standard Cyt *c* spiked in cell homogenate is represented by the dashed line.

4. Discussion

The internalization, intracellular processing and biological activity of the cargo in CPP-conjugates have not been fully deliberated. In this report, the cellular processing of conjugates of a CPP peptide, oligoarginine, and Cyt *c* prepared with either a reducible disulfide or a non-reducible thioether linkage was studied. The analysis of a reducible and cleavable disulfide versus a non-reducible and non-cleavable thioether conjugate of the cationic oligopeptide $C(R)_9$ and Cyt *c* provides us with a tool to investigate the intracellular processing of proteins in the cytoplasmic compartment. In this study, we prepared two conjugates, i.e., via a reducible disulfide bond through the cysteinyl residue of $C(R)_9$ peptide and the pyridyl disulfide group on SPDP-modified Cyt *c*, or a non-reducible thioether bond through the cysteinyl residue of $C(R)_9$ peptide and the maleimide group on SMPB-modified Cyt *c*.

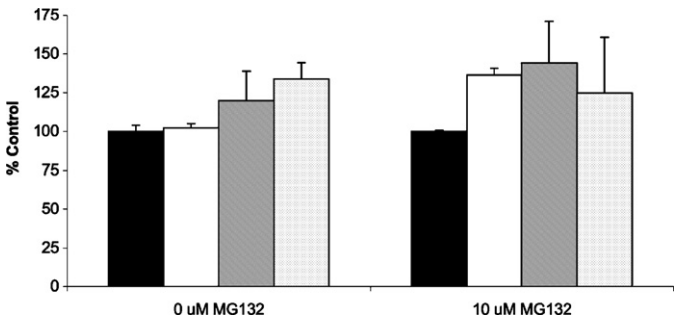

Fig. 6. MG132 effect on apoptosis. HeLa cell monolayers incubated with and without MG132 in the presence of 80 μg/mL Cyt *c* or conjugate, and analyzed by annexin V assay. PBS (closed bars), Cyt *c* (open bars), SPDP conjugate (hatched bars) and SMPB (dotted bars). Data analysis of apoptotic effect with and without MG132: PBS, SMPB, $p > 0.1$; Cyt c, $p < 0.05$; SPDP, $p = 0.1$.

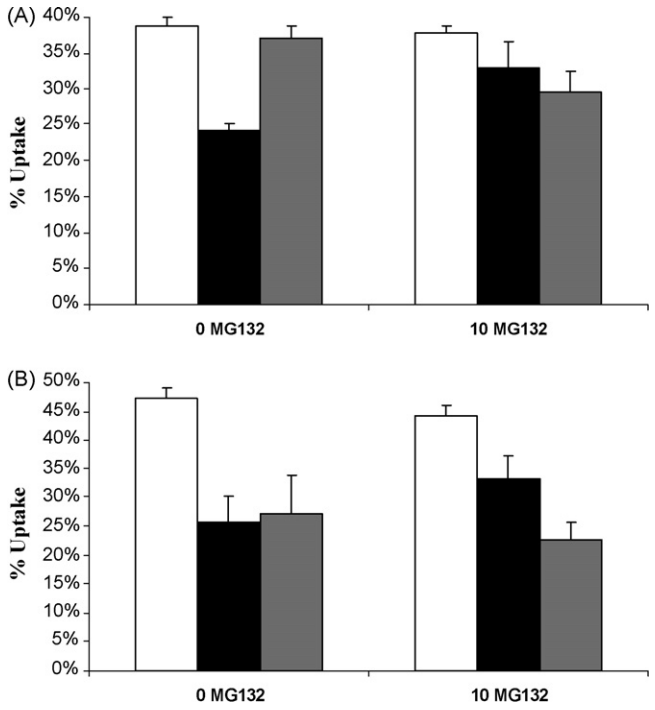

Fig. 7. MG132 effect on degradation and internalization. HeLa cell monolayers incubated with and without MG132 in the presence of 3 μg/mL Cyt *c*-$(R)_9$ conjugates, washed, trypsinized, and the cell homogenate fractionated and assayed for ^{125}I, fluorescence and protein content. Vesicular (open bars), cytosolic (closed bars), degradation (grey bars). (A) disulfide linker; (B) thioether linker. The results are presented as a percentage of total internalization (including vesicular + cytosolic + degraded). Data analysis of intracellular localization with MG132 vs. without MG132 treatment: (A) vesicular, $p > 0.5$; cytosolic and degradation, $p < 0.05$; and (B) vesicular, cytosolic, and degradation, $p > 0.1$.

The internalization and subsequent intracellular processing of the two conjugates was compared to the unmodified Cyt *c* protein in HeLa cell monolayers. It was found that the conjugation of cationic oligoarginine to Cyt *c* via either disulfide or thioether linkage resulted in a comparable enhancement in cellular uptake of Cyt *c* in HeLa cells (Fig. 1). Similar enhanced uptake was also observed in both CHO and U937 cells (data not shown). The lack of dose-dependent cytotoxicity for the conjugates suggests that there is more than one pathway, including both specific and non-specific, in their cellular uptake. However, when assayed for apoptotic effect, only the thioether conjugate, but not the disulfide conjugate or the free Cyt *c*, showed activity (Figs. 3 and 4). These findings are unexpected because it is generally believed that a disulfide linkage is required to enhance intracellular delivery of peptides and proteins as cargos to CPP-carriers (Murriel and Dowdy, 2006; Stein et al., 1999). To further elucidate the difference between these two conjugates in apoptosis, we investigated the role of proteasomes on the intracellular processing of the CPP-conjugated Cyt *c*.

The proteasome functions to control the degradation of cytosolic proteins within a cell. It is known that the release of Cyt *c* from mitochondria into the cytosol will initiate the apoptotic process by binding to cytosolic apoptosomes (Jiang and Wang, 2004). Furthermore, it has been shown that Cyt *c* is a substrate for proteasomal degradation (Huffman et al., 2003). Therefore, the involvement of proteasomes in regulating the apoptotic activity of cytosolic Cyt *c* and its conjugates was investigated. We found that when proteasomal activity in HeLa cells was inhibited by the treatment with the inhibitor, MG132, an increase of apoptotic activity in the disulfide conjugate and the free Cyt *c*, but not in the thioether conjugate or the control, was observed (Fig. 6). This observation suggests that the lack of the apoptotic activity in the disulfide conjugate is due to the degradation of released Cyt *c* by proteasomes. The finding that MG132 treatment also significantly increased the apoptotic effect of free Cyt *c* (Fig. 6) further supports the assumption that Cyt *c* is degraded intracellularly by proteasomes. The fact that the thioether conjugate was not only active in apoptosis but also was not affected by MG132, suggests that the intact Cyt *c*-oligoarginine conjugate can initiate the apoptotic cascade and can escape from proteasomal degradation. The lack of proteasomal degradation of the thioether conjugate is further supported by the finding that while MG132 decreases the degradation and increases the cytosolic localization of disulfide linked Cyt *c*, it has no effect on the thioether linked conjugate (Fig. 7). Furthermore, there was no significant increase in vesicular localization of the conjugates in MG132-treated cells (Fig. 7). Therefore, it is unlikely that MG132 had any inhibitory effect on lysosomal proteases that could result in an increase of lysosomal accumulation and, subsequently, the lysosomal escape of the endocytosed conjugates in MG132-treated cells. This finding suggests that, contrary to the general belief, the preservation of the carrier-cargo apparatus, rather than the release of the cargo, is necessary to maintain the apoptotic activity of Cyt *c*-C$(R)_9$ in the cytosol. However, the optimal linkage of Cyt *c*-CPP conjugates as described in this report may be different for other cargo attachments that are not subject to cytosolic degradation. For instance, it has been reported recently that a similar effect on pre-mRNA splicing correction was observed in peptide nucleic acid (PNA)-CPP conjugates with thioether and disulfide linkages (Abes et al., 2007).

In summary, our results suggest that the ability of Cyt *c*-C$(R)_9$ to induce apoptosis in HeLa cells depends on the membrane transduction and cytosolic processing of the conjugate. Free Cyt *c* in the cytosol, either from the cellular uptake of the native protein or from the reduction of the disulfide conjugate, will be subjected to proteasomal degradation. Consequently, the disulfide conjugate will not induce the apoptotic cascade. On the other hand, the conjugate with a thioether linkage can maintain the intact structure. Through retention of the intact conjugate, the non-reducible thioether linkage is more successful at preserving apoptotic activity of Cyt *c*, through protection of the cargo from proteasomal degradation.

Acknowledgments

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