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Biscysteine-Bearing Peptide Probes to Reveal Extracellular Thiol-Disulfide Exchange Reactions Promoting Cellular Uptake

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ABSTRACT: In recent years delivery systems based on the incorporation of thiols/disulfides have been extensively explored to promote the intracellular delivery of biological cargoes. However, it remains unclear about the detailed processes of thiol-disulfide exchanges taking place on the cell surface and how the exchange reactions promote the cellular uptake of cargoes bearing thiols or disulfide bonds. In this work, we report the rational design of biscysteine motif-containing peptide probes with substantially different ring-closing property, and how these peptide probes were employed to explore the thiol-disulfide exchanges on the cell surface. Our results show that extensive thiol-disulfide exchanges between peptides and exofacial protein thiols/disulfides are involved in the cellular uptake of these peptide probes, and importantly GSH exported from the cytosols participates extensively in the exchange reactions. We suggested that CGC-containing peptide probes can be more efficiently taken up by cells compared to other probes because of very likely the unique propensity of the CGC motif in forming doubly-bridged disulfide bonds with exofacial proteins. Our probe-based strategy provides firsthand information on the detailed processes of the exchange reactions, which would greatly benefit to the development of delivery systems based on the extracellular thiol-disulfide exchanges for intracellular delivery of biologics.

Introduction

Thiol-disulfide exchange reactions on the outer cell surface play an important role in the regulation of cell functions and the cellular entry of viruses and toxins.[1-6] For instance, accumulating evidence has implicated that extracellular thiol-disulfide exchanges regulate the assembly of extracellular matrix and the biological features associated with cancer cell invasion.[2-3] The cell infection of viruses, such as Sindbis virus and HIV, requires the reduction of critical disulfide bonds in viral envelope glycoproteins on the outer cell surface.[5-7] These extracellular exchange reactions are usually mediated by exofacial protein thiols, such as disulfide isomerases and reductases. In addition to discoveries of these natural processes, extracellular thiol-disulfide exchanges have been increasingly exploited for promoting the intracellular delivery of synthetic materials such as imaging probes, nucleic acids, peptides and proteins.[8-9] Before taking shape of this strategy (replying on extracellular thiol-disulfide exchanges) for intracellular cargo delivery, many reports have shown the beneficial effect of incorporating free thiol groups or disulfide bonds into synthetic carriers, peptides and oligonucleotides for their cellular uptake.[10-13] Until five years ago, Torres and Gait proposed conclusively to consider in future the rational modification of synthetic cargoes with thiols/disulfides to optimize the cellular delivery.[8] In recent years, many delivery systems based on incorporation of thiols/disulfides, including cysteine-rich cationic peptides, cell-penetrating poly(disulfide)s, and strained cyclic disulfides, have been developed for bioactive cargoes.[14-20]

In spite of the extensive exploration on thiols- and (mostly) disulfides-containing delivery systems, surprisingly little is known about how exchange reactions between cell surface thiols/disulfides and those in synthetic cargoes take place on the cell surface, and how these reactions can promote their cellular uptake.[8] Completely demystifying the processes of disulfide-mediated cellular uptake relies on understanding of at least the following three aspects: 1) how the extracellular thiol-disulfide exchange reactions take place; 2) which exofacial (protein) thiols participate in the exchange reactions and the formation of ultimately internalized disulfide bonds; 3) how the disulfide-bridged protein-cargo conjugates

are internalized by cells. The latter two can be explored through chemical proteomics and complementary genetic, biochemical and cell biological techniques.[17, 19] The first question, however, is difficult to be answered, because thiol-disulfide exchanges are dynamic, involving multi-steps of disulfide bond rearrangements,[21] and both exofacial protein thiols and disulfides (the predominant form) might engage in the reactions.[1, 8, 22] Moreover, small-molecule thiols, particularly glutathione (GSH) exported from cytosols, have been recently found extensively involved in dynamic thiol-thioether exchange reactions;[23] and the implication of GSH in thiol-disulfide exchanges on the cell surface is very likely. Recently, increasing evidences indicated that regulating ring tension of disulfide bonds and arrangement of cysteine residues (also numbers) in cationic peptides can achieve augmented intracellular delivery.[14-15, 18-19] In particular, pioneering works by Matile and coworkers demonstrated that increasing ring tension of disulfide bonds can considerably amplify the effect of extracellular thiol-disulfide exchanges on the cellular uptake efficiency of cargoes.[18-20] However, the thiol-disulfide exchanges taking place on the cell surface are still a "black box" not fully demystified. As an emerging principle of chemistry that can address a central challenge in life sciences in regarding to the delivery of bioactive species,[8, 24] such an exploration on thiol-disulfide exchanges behind the principle is important, not only to our understanding of the complex extracellular behavior, but to the development of more efficient delivery strategies for biological cargoes (e.g., oligonucleotides, peptides and proteins).

In this work, by taking advantage of the unique feature of the CGC motif (cysteine-glycine-cysteine) in forming the ring-closed disulfide bond, compared with the CGGC and CC motifs (Figure 1a),[25-28] a series of cell-penetrating and fluorescent probes were designed and examined to explore thiol-disulfide exchange reactions taking place on the cell surface. CGC-containing cationic peptides exhibit ~20–50-fold increases in cellular uptake efficiency as compared to their non-cysteine analogs, and ~3–10-fold increases as compared to CGGC/CC and single cysteine analogs, implying the beneficial effect of the unique ring-closing/opening rearrangement of disulfide bonds on cellular uptake. By correlating the difference of cellular uptake efficiency to the different ring-closing property of the biscysteine motifs, the

extracellular thiol-disulfide exchange reactions promoting the cellular uptake can thus be reliably deduced (Figure 1a). Moreover, our findings indicate the vital role of exported GSH in mediating the thiol-disulfide exchanges between reducing CGC, ring-closed CGC, CGC-GSH mixed disulfides, and CGC-exofacial protein conjugates. We demonstrated, for the first time, that the enhanced cellular uptake mediated by CGC motif stems from the increased possibility of the reactions of cell surface protein thiols and disulfides with CGC-related species circulating in the extracellular environment to form pre-internalized exofacial protein-linked products, and the larger propensity of forming double disulfide-bridged conjugates with exofacial proteins (relative to CC and CGGC).

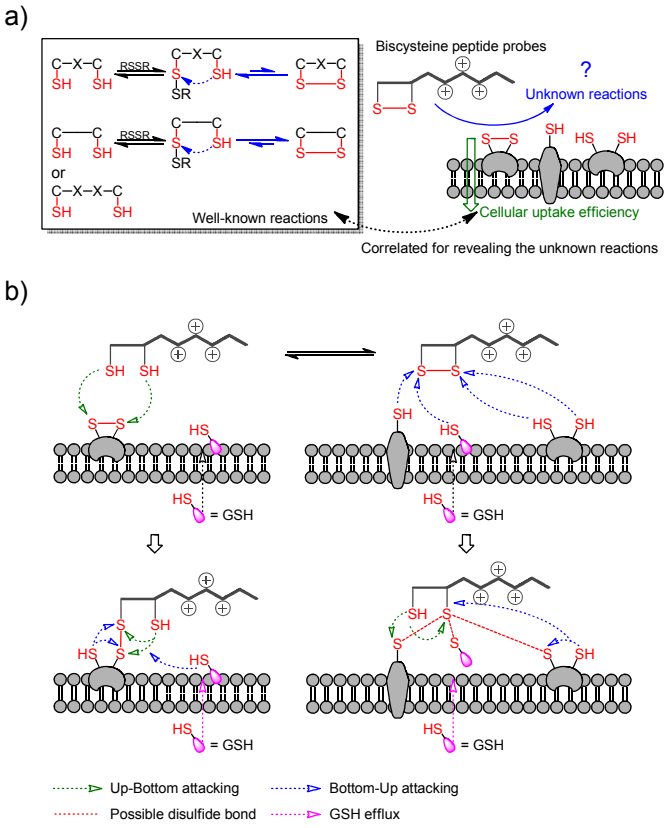

Figure 1. (a) Unique properties of $C(X)_{n=0,1,2}C$ (X: any amino acid residue except cysteine) motifs in thiol-disulfide exchanges. Ring-closed CXC motif is structurally more constrained than ring-closed CC and CXXC motifs, thus the CXC motif tends to form mixed disulfide bonds with other thiols while the others are in favor of forming ring-closed disulfide bond. Extracellular thiol-disulfide exchange reactions will be deduced by correlating the difference of cellular uptake efficiency of biscysteine-bearing peptide probes to the ring-closing property of $C(X)_{n=0,1,2}C$ motifs. (b) Conceivable thiol-disulfide exchange reactions between biscysteine-containing cationic peptides and cell-originated thiols and disulfides taking place on the cell surfaces. The reactions were divided into two categories: 1) up-bottom attacking of disulfide bonds by biscysteine thiols (green dash lines); 2) bottom-up attacking of disulfide bonds by exofacial protein thiols or GSH (blue dash lines). Red dash lines represent possible disulfide bonds formed between peptides and cell-surface associated thiols (i.e., exofacial proteins and exported GSH).

Experimental Section

Materials and Instruments. Peptides labeled with 5-carboxyfluorescein were purchased from KE Biochem Co., Ltd (Shanghai, China) with >95% purity. Analytical chromatograms and mass spectra were used to confirm the purity and identity of peptides. Hela, MCF7, CHO and RAW 264.7 cells were purchased from CoBioer Biosciences Co., Ltd. (Nanjing, China). Conventional cell culture reagents such as Dulbecco's Modified Eagle Medium (DMEM) and phosphate buffered saline (PBS) were obtained from Thermo Scientific (Beijing, China). Anti-PDI antibody and Alexa Fluor 568 goat anti-mouse IgG (H+L) were purchased from Abcam. Inhibitors of Endocytosis/macropinocytosis (wortmannin and cytochalasin D) and PDI inhibitor (bacitracin) were acquired from Cayman Chemical and Selleck, respectively. Cell organelles tracer reagents, 4',6-diamidino-2-phenylindole (DAPI), LysoTracker Red DND-99 and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), were purchased from Thermo Scientific (Beijing, China) or Beyotime. Other reagents including 5,5'-dithiobis (2-nitro-benzoic acid) (DTNB), 2,2'-dithiodipyridine (DTDP), L-cysteine (Cys), glutathione (GSH), dithiothreitol (DTT) were purchased from Sigma-Aldrich. Eppendorf tubes (1.5 mL), 6-well chambers and cell culture dishes were purchased from JET BIOFIL (Guangzhou, China). Glass bottom cell culture dishes were purchased from Nest Biotechnology Co., Ltd. (Wuxi, China). Analytical and semipreparative HPLC (Shimadzu systems) was served to identify and purify peptides. Bruker MicroFlex matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) was used to characterize peptides. Fluorescent images were carried out on a Leica TCS SP5X confocal microscope system. Fluorescent signals in cells were analyzed by a flow cytometry (BD FACSAria II).

Synthesis of peptide probes. Ring-closed peptide probes were synthesized by a direct oxidation of the cysteine-bearing peptides in 20% aqueous DMSO for 24 h. Mono-disulfide analogs **2**-TP and **2**-SG were obtained by reacting **2** with excessive amounts of DTDP and oxidized GSH (GSSG) in 20% aqueous DMSO. Bioreduction-tolerant dimer (**2**-M-**2**) were obtained by reacting **2** with bismaleimide cross-

linker with the molar ratio of 2:1 in 20% aqueous DMSO overnight. All of these target products were purified by HPLC and characterized by MALDI-TOF-MS (See Figures S1–S8).

Cell Culture. Hela cells (or MCF7, CHO and RAW 264.7 cells) were maintained in DMEM medium (high glucose) supplemented with 10% FBS and 1% penicillin/streptomycin (penicillin, 10000 U/mL; streptomycin, 10000 U/mL) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were passaged at about 80% cell confluency using a 0.25% trypsin solution.

Analysis of cellular uptake of peptide probes. The efficiency of cellular uptake of peptide probes into the cell was analyzed by a flow cytometry. Cells were seeded in a 6-well plate at 2×10^5 cells/well for Hela, CHO, RAW 264.7 cells and 5×10^5 cells/well for MCF7 cells prior to experiments. After incubation for 24 h to make cells adhere, 1 μM or different concentrations (0.2 – 5 μM) of peptide probes in DMEM containing 10% FBS were added. For purpose of verifying the thiol-disulfide exchange reaction really happen on the cell surface, 2 mM DTT was used to pretreat cells for 10 min to activate the thiol on the cell membrane while 2 mM DTNB co-incubated with cells was served for blocking them. To investigate whether the uptake pathway of ring-closed **4** into the cell belongs to the traditional endocytosis, 100 nM cytochalasin D or 50 nM wortmannin were co-incubated with cells to inhibit the endocytosis; to explore the influence of temperature, cells were incubated with ring-closed **4** in a 4 degrees freezer; with the purpose of finding out whether ring-closed **4** passed through the lysosome or not, 150 μM chloroquine was used to increase the permeability of lysosomes at the time of ring-closed **4** had incubated with cells for 4 or 24 h. After the incubations at 37 ^{o}C, 5% CO_2, the cells were thoroughly washed three times with PBS, and then detached by a treatment of 0.25% trypsin solution and collected by centrifugation. Finally, the cells were resuspended by cold PBS. Fluorescent signals in cells were detected using a flow cytometry (BD FACSAria II), and at least 10000 events of live cells were collected each analysis. All experiments were conducted in triplicate. All of the reagents were not cytotoxic at the concentrations used.

Analysis of the distribution of peptide probes in the cell. The distribution of peptide probes in cells was spied on a Leica TCS SP5X confocal microscope system. Hela cells were plated at a density of 10^5 cells/well into a glass bottom cell culture dish. After incubation for 24 h to make cells adhere, 1 μM peptide probes (i.e., **2**-TP or ring-closed **4**) in DMEM containing 10% FBS were incubated with cells at 37 ^{o}C, 5% CO_2 for 1 or 4 h. In order to track the different cell organelles, the commercial tracer reagents were used, that is DAPI for cell nucleus, LysoTracker Red DND-99 for lysosome and DiI for cell membrane, all of them were used according to the instruction manual specification, and cells were fixed with paraformaldehyde for dyeing nuclear while not fixed for dyeing cell membrane. Multi-channel imaging was carried out on a Leica TCS SP5X confocal microscope system (DAPI channel: λ_{ex} = 405 nm, λ_{em} = 420–480 nm; FAM channel: λ_{ex} = 488 nm, λ_{em} = 520−650 nm; DND-99 channel: λ_{ex} = 575 nm, λ_{em} = 595−750 nm; DiI channel: λ_{ex} = 543 nm, λ_{em} = 560−650 nm).

Characterization of transformation between reducing and ring-closed peptides during cell culture. Hela cells were seeded in a 6-well plate at 1× 10^6 cells/well, after incubation for 24 h at 37 ^{o}C, 5% CO_2, the medium was removed and the cells were washed with PBS. Cells were then incubated with 10 μM peptide probes in PBS (10 mM, pH=7.4) for 30 min, and then peptides remaining in the buffer were analyzed using HPLC (Waters Xbridge C18 column (4.6 mm × 250 mm, 5 μm), 1.0 mL·min^{-1} flow rate, isocratic with 5 vol% acetonitrile (ACN) + 0.1% trifluoroacetic acid (TFA) for 5 min followed by a linear gradient of ACN + 0.1% TFA (5–80 vol%) over 30 min).

Results and Discussion

Design principle for peptide probes. We launched our studies on the basis of a preknowledge that CXC motif (cysteine-any-cysteine), compared with CXXC and CC motifs, is more oxidation-resistant to form the ring-closed disulfide bond and possess a propensity of forming mixed mono-disulfides with other thiols (Figure 1a).[25, 27] Mixed mono-disulfides between CXXC (or CC) and other thiols are extremely unstable, because the other cysteine thiol can facilely attack the mixed disulfide bond and results in the formation of ring-closed disulfide bond.[29-30] Given that the efficiency of the cellular uptake of peptides is closely correlated with the formation of mixed disulfide bonds between peptides and exofacial proteins, the extracellular thiol-disulfide exchange reactions can thus be reliably deduced by correlating the difference of cellular uptake efficiency of biscysteine-bearing peptides to the ring-closing property of biscysteine motifs (Figure 1a). To facilitate the analysis of thiol-disulfide exchange reactions on the cell surface, we divided the reactions into two categories: 1) up-bottom attacking of disulfide bonds by the biscysteine thiols; 2) bottom-up attacking of disulfide bonds by exofacial protein thiols or GSH (Figure 1b; reactions are very complex, albeit drawing in a simplified form).

To facilitate the thiol-disulfide exchanges between peptides and cell surface thiols, and to amplify the thiol/disulfide-mediated effect of cellular uptake, we appended a cationic sequence (RKKRRQRRR; cell-penetrating Tat)[31-32] to the $C(G)_{n=0,1,2}C$ motifs (**3**–**5**; Figure 2a), which has been found very appropriate for mediating the extracellular thiol-disulfide exchange reactions.[33] The ring-closed disulfides were prepared by direct oxidation in aqueous buffers and purified by HPLC. The mono-cysteine-containing peptide (**2**) and its cysteine mutant (**1**) were designed and synthesized for comparison, and the mono-disulfide analog was prepared by reacting **2** with 2,2'-dithiodipyridine (DTDP) or oxidized GSH (GSSG) (i.e., **2**-TP and **2**-SG). In addition, all peptides were labeled with 5-carboxyfluorescein (FAM) to enable the analysis of cellular uptake through flow cytometry and confocal laser scanning microscopy (CLSM).

Cellular uptake of peptide probes. Cellular uptake of oxidized peptides (**2**–**5**) and the control (**1**) was measured in four cell lines (Figure S9), including human cancer cells (Hela and MCF7), Chinese hamster ovary (CHO) cells, and murine macrophages (RAW 264.7) cells. After treatment of cells with 1 μM peptide probes for 4 h, external peptides were washed away and the fluorescence intensity of individual cells was recorded by flow cytometry. As previous studies have shown that Tat might bind onto cell-surface proteoglycans, we used the verified trypsin treatment to remove the surface-bound probes.[11, 34] For all cell lines examined, **2**-TP can be more efficiently taken up than **1**, like that observed from other reports,[9] indicating the benefit of conventional disulfide bonds. Remarkably, the uptake of **4** (i.e., the CGC-containing probe) is 20–50-fold higher than **1**, or 3–10-fold higher than **2**, **3**, and **5** (the difference in uptake between **2**, **3**, and **5** was not significant; Figure 2b). CLSM images of Hela cells treated under the same conditions are in agreement with the results from flow cytometry (Figure 2c). Moreover, the CLSM images clearly show that fluorescent regions are mainly located at the cytosols, and fluorescence signals from cell surface are not predominant, suggesting the successful internalization of the probes by cells. To further verify the unique feature of the CGC motif, **1**, **2**-TP, and **4** of different concentrations (0.2–5 μM) were incubated with Hela cells, and the fluorescence intensity of cells was recorded by flow cytometry (Figure 2b). At all concentrations examined, the cellular uptake of **4** is remarkably more efficient than **1** and **2**-TP, and a maximum enhancement of ~40 folds was achieved at a concentration of 1.0 μM.

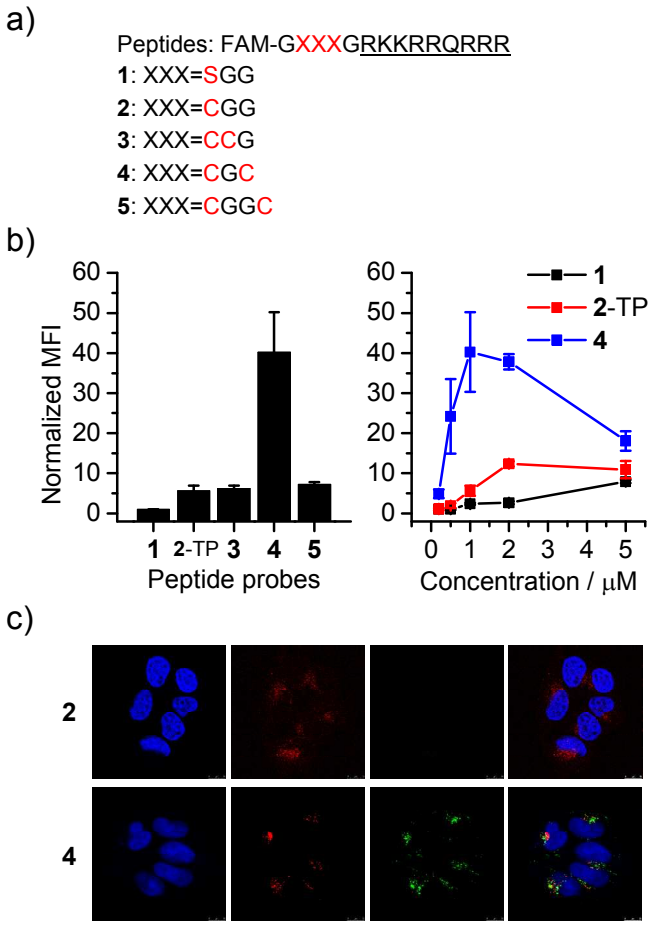

Figure 2. (a) Sequences of peptide probes. Peptides (**1**–**5**) were N-terminally labeled with 5-carboxyfluorescein (FAM) and C-terminally amidated. (b) Flow cytometry data showing the fluorescence of HeLa cells after incubation with 1 μM **1, 2**-TP and ring-closed **3**-**5** (left) or various concentrations (0.2–5 μM) of **1**, **2**-TP and ring-closed **4** (right) in 10% FBS medium for 4 h. MFI: mean fluorescence intensity. MFI for each sample was normalized to a control sample treated with peptide **1**. Data are presented as mean ± s.d. (n = 3). (c) Confocal fluorescence images of Hela cells incubated with 1 μM **2**-TP (top) and ring-closed **4** (bottom) in 10% FBS medium for 4 h. DAPI was used to track the cell nuclei and LysoTracker Red DND-99 was used to track lysosomes; green fluorescence was from the FAM-labeled peptides.

Extracellular thiol-disulfide exchanges. Cellular uptake of peptide probes (**2**-TP, **4**, and **5**) can be further activated and inhibited, respectively, when cells were pretreated with dithiothreitol (DTT) or coincubated with Ellman's reagent (DTNB), (Figure 3a). Treatment of cells with DTT converts exofacial protein disulfides into thiols, while incubation with DTNB results in oxidation of exofacial protein thiols to disulfides.[35-36] Thus, this result demonstrates that the bottom-up attacking of disulfide bonds in the peptide probes by exofacial protein thiols is decisive to the enhanced cellular uptake of **2**–**5** relative to **1**. Moreover, as the bottom-up thiol-attacking is unified for all probes, the difference in efficiency of cellular uptake is considered arising from the difference in pathways of the subsequent up-bottom attacking of disulfide bonds by the newly-released cysteine thiol (for the $C(G)_{n=0,1,2}C$-appending peptides). However, it is not clear how the subsequent thiol-disulfide exchange reactions take place, and how they determine the ultimate cellular uptake of peptides. To explore this, first we examined and compared the cellular uptake of reducing peptides **2**–**5** under the same condition as that for their oxidizing forms, because in this situation thiol-disulfide exchanges on the cell surface is initiated by the up-bottom attacking of exofacial protein disulfides by bis-cysteine thiols of the $C(G)_{n=0,1,2}C$ motif, a process that is in sharp contrast to that was taken by oxidizing peptides. Quite surprisingly, there is no significant difference in cellular uptake efficiency between reducing and oxidizing peptides (Figure 3b). It is particularly noteworthy that the CGC-bearing probe (**4**), both in the reducing and the oxidizing form, can be equivalently taken up by cells with an efficiency surpassing all other cysteine-containing peptides by >3 folds. This result has a strong implication that extensive thiol-disulfide exchanges between the peptide probes and exofacial proteins might take place on the cell surface, resulting in the formation of identical mixed disulfides between peptide probes and exofacial proteins for both reducing and oxidizing peptides—that is, the disulfide-bridged complexes ultimately mediating the cellular uptake.

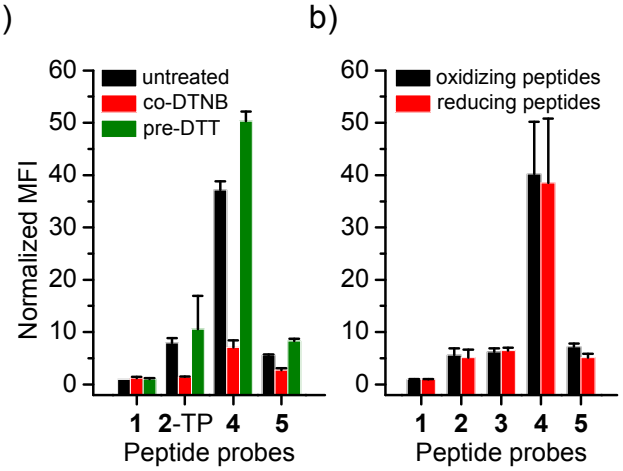

Figure 3. (a) Normalized MFI showing cellular uptake of peptide probes when cell surface-thiols were either inhibited or activated. Untreated: cells treated with 1 μM **1**, **2**-TP, and ring-closed **4** and **5** for 4 h (dark); Co-DTNB: cells coincubated with 2 mM DTNB when treated with peptides (red); Pre-DTT: cells pretreated with 2 mM DTT before treatment with peptides (green). (b) Cellular uptake of **1**-**5** into cells under oxidizing (dark; of note, oxidizing **2** is **2**-TP.) and reducing forms (red), respectively; concentration of peptides: 1 μM. MFI: mean fluorescence intensity. MFI for each sample was normalized to a control sample treated with peptide **1**. Data are presented as mean ± s.d. (n = 3).

To collect more evidences to support the above deduction, we then examined the products that formed during the extracellular thiol-disulfide exchanges by LC-MS analysis of the incubation medium. When reducing peptides (**3**–**5**) were incubated with cells, we observed both reducing peptides and ring-closed disulfides (Figure 4), suggesting that the rapid conversion from reducing peptides to oxidizing/ring-closed peptides on the cell surface. Interestingly, we found that there is remarkably less amounts of both reducing and oxidizing **4** remaining in solutions compared to the other two peptides (**3** and **5**), in agreement with the superior cellular uptake of **4**. It is more noteworthy that when **2**-TP was incubated with cells, we observed a rapid and complete conversion of the peptide into the peptide/GSH-mixed disulfide (**2**-SG) (Figure 4d). This finding implies that GSH exported from the cytosols is extensively implicated into the extracellular thiol-disulfide exchanges, which is essentially in agreement with previous findings in cell surface thiol-mediated thioether exchange reactions.[23] GSH is able to react di-

rectly with peptide disulfides or indirectly react with cell surface-bound peptides (i.e., exofacial protein/peptide mixed disulfides) during and/or after the GSH effusion to afford the GSH-mixed disulfides. This result also stresses the importance of considering the implication of the GSH efflux in analyzing the interconversion between reducing and oxidizing $C(G)_{n=0,1,2}C$-bearing peptides and the thiol-disulfide exchange reactions of these peptides with exofacial protein thiols and disulfides.

The involvement of disulfide exchanges with GSH efflux, together with the remarkable difference in efficiency of cellular uptake for cysteine-bearing peptide probes (**2**–**5**), allow us to more reliably deduce the thiol-disulfide exchange reactions taking place on the cell surface. This is also one of the core objective goals of exploring peptide probes in this study. It is reasonable to assume that these species coexist on the cell surface, including proteins with an isolated monothiol or closely spaced dithiols,[37-38] proteins with both a monothiol and a GSH-mixed disulfide, and protein disulfides (Figure 1b). The CGC-bearing peptide substantially differs from CC- and CGGC-containing peptides in thiol-disulfide exchanges due to the unique propensity of CGC motif in the formation of doubly-bridged disulfides with exofacial proteins with the assistance of disulfide exchanges with GSH (Figure 5). First, the oxidizing CGC motif would react with exofacial proteins bearing both a monothiol and a GSH-mixed disulfide to afford the doubly-bridged disulfides via two consecutive disulfide exchanges (Figure 5, route a). In contrast, the CC/CGGC motif would detach from the monodisulfide-bridged intermediate due to the propensity of reforming the ring-closed disulfide bond. Secondly, the overall lifetime of exofacial protein-CGC conjugates linked by a monodisulfide bond in the presence of vicinal thiol(s) would be longer than their CC/CGGC-counterparts, as a result, earning more time for the subsequent formation of the doubly-bridged disulfides through either direct oxidation of the vicinal thiols or multistep disulfide exchanges, which competes with the ring-closing exchange reactions (Figure 5, route b). Thirdly, the newly-formed CGC-GSH mixed disulfides, either from reactions between oxidizing CGC and GSH efflux or from the cleavage of monodisulfide-bridged intermediates by GSH, would also be longer-lived than their CC/CGGC-counterparts, which enable their direct thiol-disulfide exchanges with exofacial protein di-

sulfides to take place more efficiently to afford the doubly-bridged disulfides (Figure 5, route c). Finally, we have to stress that the frequently-involved doubly-bridged disulfides, as compared to the conventional single disulfide bond, should be substantially more stable, on the one hand due to the absence of vicinal thiols capable of cleaving the vicinal disulfide bond, and on the other hand due to the enhanced stability against GSH reduction resulting from the cooperativity of the two disulfide bonds.[25] An evidence to support this deduction also comes from the observation of the rapid conversion of **2**-TP to **2**-SG on the surface of cells (Figure 4d).

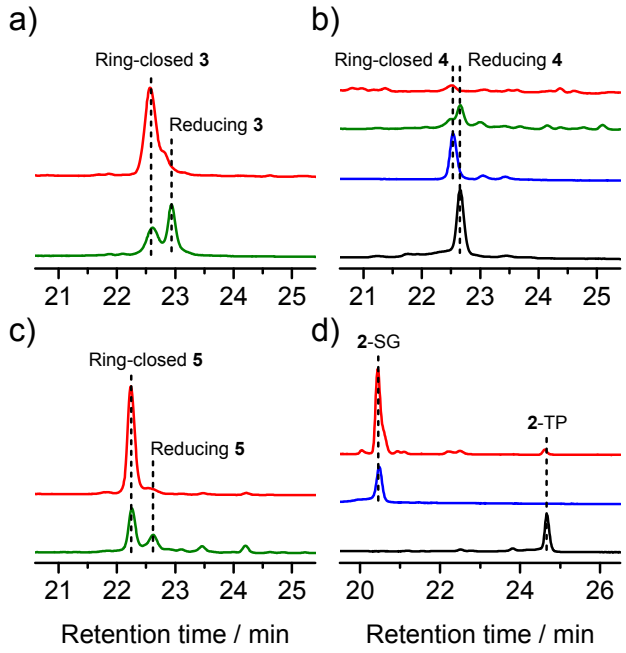

Figure 4. HPLC chromatograms showing the transformation between reducing and oxidizing/ring-closed peptides during cell culture. (a) Products observed after the incubation of reducing **3** (10 μM; green line) and ring-closed **3** (10 μM; red line) with cells for 30 min, respectively. (b) Products observed after the incubation of reducing **4** (10 μM; green line) and ring-closed **4** (10 μM; red line) with cells for 30 min, respectively. Black and blue line shows HPLC peaks of standard purified peptides. (c) Products observed after the incubation of reducing **5** (10 μM; green line) and ring-closed **5** (10 μM; red line) with cells, respectively. (d) Conversion of **2**-TP into **2**-SG observed after 30 min of incubation (red line); black and blue line shows HPLC peaks of standard purified peptides.

Extracellular protein disulfide isomerase (PDI) has been shown to mediate the reduction of disulfides on the cell surfaces, meanwhile modulating the cell-surface density of free thiols.[39-41] However, PDI appears to be not involved in the processes of the CGC-mediated cellular uptake, because treatment with either a PDI inhibitor (bacitracin) or an anti-PDI antibody (i.e., coincubated with **4**) exerted no effect on the uptake (Figure S10).[39] Moreover, even though the cells were pretreated with DTNB for 0.5 h to temporarily block the cell-surface thiols, coincubation of the antibody with **4** still did not affect the cellular uptake of the peptide. Further colocalization analysis revealed that the peptide **4** bound on the cell surfaces (0.5 h after the incubation; before entering into cells) is negligibly colocalized with the cell-surface PDI (Figure S11). These results implies very likely that when the primitive thiol-disulfide exchanges on the cell surfaces were extensively inhibited (by the PDI inhibitor, anti-PDI antibody, and DTNB), other inside-out redox stimuli, such as the aforementioned GSH export, was still able to initiate thiol-disulfide exchange reactions between CGC-bearing peptides and exofacial protein thiols/disulfides without obvious compromise.

Internalization of peptides into cells. Further studies on the internalization of **4** into cells indicate that the CGC-mediated cellular entry of peptides was insensitive to standard endocytosis/macropinocytosis inhibitors such as wortmannin and cytochalasin D (Figure 6a), but extremely sensitive to cell culture temperature. At 4 °C, the efficiency of cellular uptake was markedly reduced. These results are in agreement with other reports on exofacial thiol-mediated cellular uptake, suggesting a new and energy-dependent uptake pathway not completely revealed.[16, 18] Time-sequential analysis of cell fluorescence indicates that the peptides were bound onto the cell membranes first (~1 h, Figures S12 and S13), and subsequently internalized into the cytosols (~4 h incubation). Moreover, the intracellular fluorescence (from **4**) was not colocalized with LysoTracker Red DND-99 tracked lysosomes, partially because the labeled FAM is weakly fluorescent under acidic conditions. This result suggests that the fluorescence intensities recorded previously by flow cytometry or CLSM (after ~4 h incubation) mainly comes from peptides located outside the endosomes and the lysosomes. To further confirm if there were peptides

resided in the endosomes/lysosomes, the cells (incubated with **4** for 4 h and 24 h, respectively) were further treated with chloroquine, a known pH-disrupting agent for endosomes and lysosomes,[36] to enhance their membrane permeability. Interestingly, no significant change in the fluorescence intensity was observed for the cells pretreated with peptides for 4 h, but after 24 h treatment, we observed a marked increase in fluorescence (~ 4-fold) (Figure 6a), implying the presence of mixed cellular uptake pathways. The peptides can initially escape the capture by lysosomes or be efficiently taken up without involvement of endosome/lysosome-dependent pathways; however, in the case of extended periods of incubation, an uptake pathway to endosomes or lysosomes indeed exists, though there are still >20% peptides (after 24 h incubation; evaluated by fluorescence intensity) successfully delivered to other intracellular spaces remaining to be further characterized.

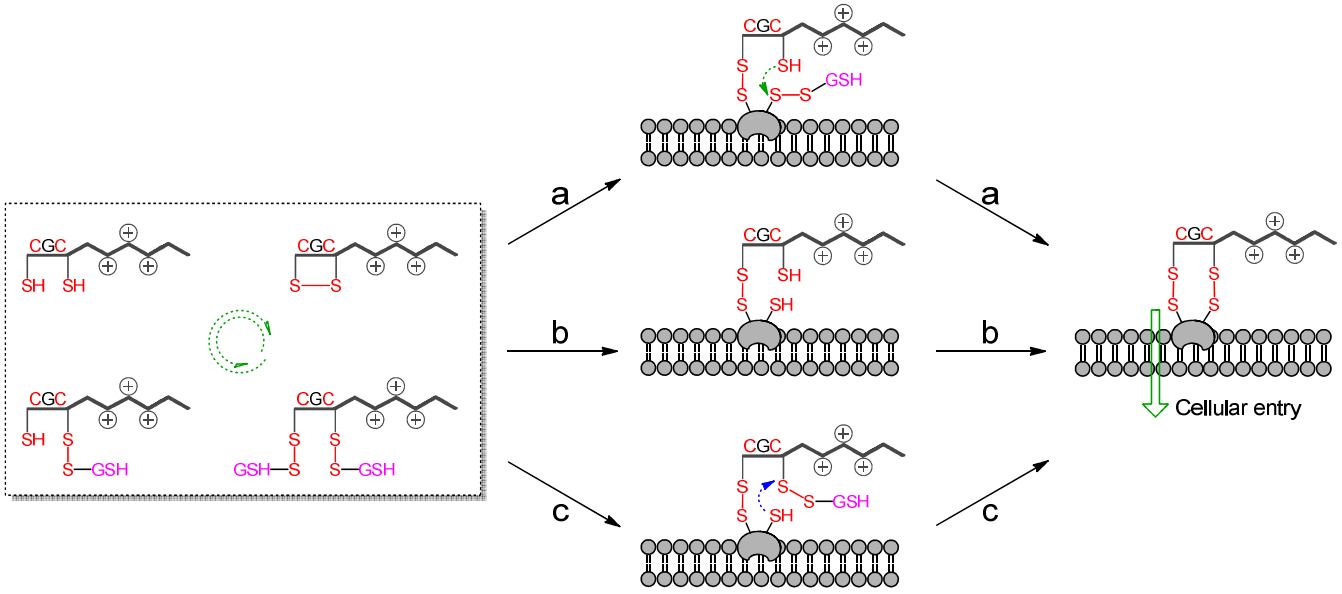

Figure 5. Several routes of forming doubly-bridged disulfides between CXC-bearing peptides and exofacial proteins; CGC-bearing peptides substantially differ from CC- and CGGC-containing peptides in thiol-disulfide exchanges due to the unique propensity of the CGC motif in forming doubly-bridged disulfides with exofacial proteins with the assistance of GSH. Extensive thiol-disulfide exchanges between peptide probes and cell-surface thiols and disulfides lead to formation of CGC-GSH mixed disulfides shown in the box.

Generality of the proposed mechanism. We next demonstrated that the demystified thiol-disulfide exchange reactions taking place on the cell surface are not restricted to the Tat sequence; instead, the reactions can be applied to other peptides. Peptides **6**–**8** bearing three arginine residues (RRR; to facilitate cell-surface association) were designed and synthesized (Figure 6b), and their cellular uptake were examined through flow cytometry. CGC-bearing **8** can be significantly more efficiently taken up by cells compared to its non- and mono-cysteine analogs (**6** and **7**) (Figure 6b). These results suggest that specific cell-associated functions of peptides, e.g., cell-penetrating property of the Tat sequence, are not required for thiol-disulfide exchange reactions to take place on the cell surfaces, and the cationic property of peptides merely accelerates the exchange reactions via attractive electrostatic interactions between peptides and cell membranes. Indeed, we observed no effect of the CGC motif on the cellular uptake of a negatively charged peptide (Figure S14).

One question worthy of being further discussed is that the CGC-bearing peptides might dimerize on the cell surface as a consequence of the complicated thiol-disulfide exchange reactions on the cell surface,[25] which might contribute, at least to some extent, to the enhanced cellular uptake of CGC-bearing peptides, because Tat dimers can indeed be significantly more efficiently taken up by cells compared to its monomer.[42] This conjecture is difficult to be dismissed completely. However, the following observations suggest that the dimerization of peptides should not play a predominant role in promoting the cellular uptake of CGC-bearing peptides. First, the cellular uptake of CGC monomer (**4**) is obviously more efficient than mono-disulfide-bridged **2** (Figure S15). Secondly, even when **2** was dimerized through a stable (bioreduction-tolerant) bismaleimide crosslinker, the efficiency of cellular uptake is still significantly lower than that of CGC dimers (**4=4**) (Figure S16), implying strongly that some other factors, e.g., the thiol-disulfide exchanges, rather than the dimerization itself, should contribute to the superior cellular uptake of the CGC-bearing peptides. Indeed, we found that when exofacial protein thiols on the cell surface were blocked by incubation with DTNB, the efficiency of cellular uptake of **4=4** was significantly decreased (Figure S16), though in this case the bioreduction of CGC dimers to monomers on the

cell surface was drastically quenched. In contrast, the cellular uptake of the stable dimeric peptide **2** (i.e., bioreduction-tolerant **2**-M-**2**) was not affected by the DTNB incubation. These results also stress the important role of the dimeric CGC motif in the cellular uptake of **4=4**, which would facilitate the uptake by initiating the similar thiol-disulfide exchange reactions happened to CGC monomers. In addition, the formation of **4=4** was not observed during the cell culture, as monitored by LC-MS.

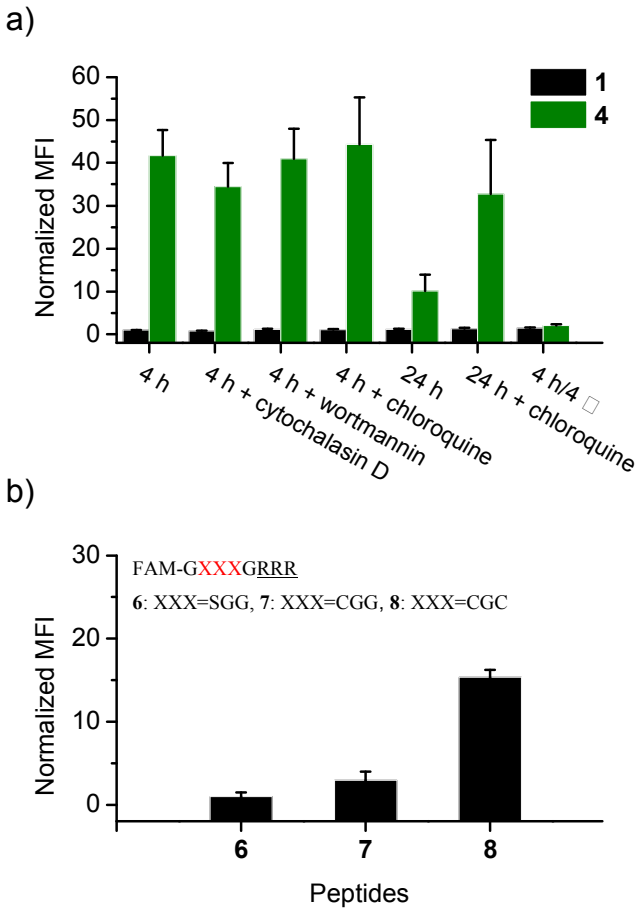

Figure 6. (a) Flow cytometry data showing the fluorescence of HeLa cells after incubation with 1 μM **1** (dark) and ring-closed **4** (green) in 10% FBS medium. 4 h: cells incubated with peptides (and inhibitors) for 4 h; 24 h: cells incubated with peptides for 24 h. 100 nM Cytochalasin or 50 nM wortmannin was coincubated with cells to inhibit the endocytosis; 150 μM chloroquine was added into the medium to increase the permeability of lysosomes when cells have been incubated with peptides for 4 h and 24 h, respectively. 4 ^{o}C: low temperature inhibition of cellular uptake. MFI for each sample was normalized to a control sample treated with peptide **1**. Data are presented as mean ± s.d. (n = 3). (b) Sequences of peptides **6**–**8**. Peptides were N-terminally labeled with FAM and C-terminally amidated. Flow cytometry data showing the fluorescence of HeLa cells after incubation with peptides in 10% FBS medium for 4 h (dark). MFI for each sample was normalized to a control sample treated with **6**.

Conclusions

In summary, we report the rational design of biscysteine motif-containing peptides with substantially different ring-closing property, and how these peptide probes were employed to explore the thiol-disulfide exchanges on the cell surface. CGC motif-containing peptides exhibit a significantly higher efficiency of cellular uptake compared to peptides with a CC and CGGC motif and a mono-cysteine, indicating that some thiol-disulfide exchange reactions not yet known which facilitate the formation of exofacial protein-CGC mixed disulfide bonds would take place on the cell surface. Further examination revealed that interactions between peptides and exofacial protein thiols/disulfides involve extensive thiol-disulfide exchanges before the formation of the ultimate complexes for cellular uptake, and more importantly the involvement of GSH efflux in the exchange reactions were demonstrated. By systematically comparing the ring-closing property of CGC motif with CC and CGGC motif, the results obtained from our probes further suggest that the superior cellular uptake of CGC-bearing peptides, compared to other peptides, arises from very likely the unique propensity of CGC motif in the formation of doubly-bridged disulfides with exofacial proteins, a process that is essentially fueled by extensive exchanges of reducing, oxidizing and GSH-/exofacial protein-mixed CGC-relative species, and the intrinsically unfavorable ring-closing propensity of CGC motif. These facts are very difficult to be uncovered without exploiting potent molecular probes due to the lack of efficient *in situ* techniques for characterization of real-time dynamic reactions on the cell surface. The thiol/disulfide-mediated cellular uptake, as a promising delivery strategy developed on the basis of pure chemical principle, has been proved to be effective for the delivery of biological cargoes that are extremely membrane-impermeable. This study would provide an important foundation for further more rationally taking advantage of thiol-disulfide exchanges on the cell surface for the development of intracellular delivery systems.

ASSOCIATED CONTENT

Supporting Information.

MS characterization of peptides and supplementary data (Figures S1–S16) regarding the CGC-mediated cellular uptake of peptides for different cells lines, the role of PDI on the cell surface, the internalization process of CGC-mediated cellular entry, the cellular uptake of negatively charged peptides, and the discussion on the dimerization of peptides on the cell surface. This material is available free of charge via the Internet at http://

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Additional information

The authors declare no competing financial interests.

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