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Ribosomal Synthesis of Peptidase-Resistant Peptides Closed by a Nonreducible Inter-Side-Chain Bond

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ABSTRACT Here we report a new enabling technology for the synthesis of peptidase-resistant cyclic peptides by means of genetic code reprogramming involving the flexizyme (a tRNA acylation ribozyme) and PURE (a reconstituted cell-free translation) systems. In this work, we have developed a new nonproteinogenic amino acid bearing a chloroacetyl group in the side chain, which forms a physiologically stable thioether bond by intramolecular reaction with the sulfhydryl group of a Cys residue in the peptide chain upon translation. Significantly, this chemistry takes place spontaneously *in situ* of the translation solution, giving the corresponding cyclic peptides independent of ring sizes. We have used this method to convert human urotensin II, known as a potent vasoconstrictor, to its analogue containing a thioether bond, showing that this new analogue retains biological activity. Moreover, this peptide exhibits remarkable resistance against peptidases under reducing conditions. Thus, this technology offers a new means to accelerate the discovery of therapeutic peptidic drugs.

A large number of naturally occurring peptides are known to exhibit biological activity. Many of these peptides are constrained by a cyclic scaffold (*1–3*), which not only increases their affinity to the biological targets but also improves their physiological stability against peptidases (*4, 5*). Therefore, such a structure would be an important feature for developing therapeutic agents. In nature, cyclic peptides are generally synthesized in two distinct ways. Microbial macrocyclic peptides are synthesized by nonribosomal peptide synthetase (NRPS) comprised of multiple enzymes, among which a carboxy-terminal thioesterase generally catalyzes the cyclization (*5, 6*). NRPS can synthesize cyclic peptides with a variety of cyclization formats, such as N–C-termini amide bond closure and side chain–C-terminus depsi bond closure (*7*). Moreover, those synthesized by NRPS often contain unique nonproteinogenic amino acids (*8*). The combination of these two features of macrocyclic peptides confers their stability against peptidases. A serious drawback of the NRPS system is the difficulty of its engineering; several studies have been reported to generate novel peptides by manipulating NRPS, but it is still laborious to design and synthesize cyclic peptides by this method (*9–11*).

Another way of generating cyclic peptides is mRNA-directed synthesis using the translation apparatus. When a linear peptide containing two or more cysteine (Cys) residues is expressed in accordance with its mRNA template, a disulfide bond(s) is formed by oxidation to yield the corresponding cyclic peptide (*12*). Since any sequence of template DNA can be readily prepared by solid-phase chemistry, its transcription followed by translation produces the desirable cyclic peptide, thus

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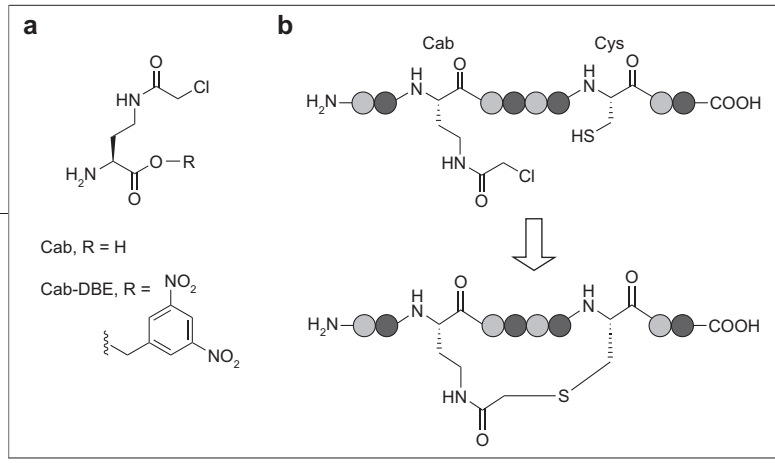

Figure 1. Incorporation of Cab into a peptide chain followed by inter-side-chain cyclization. (a) Chemical structure of N^{γ}-(2-chloroacetyl)-α,γ-diamino-butylic acid (Cab) and 3,5-dinitrobenzyl ester (DBE) form of Cab as a substrate for dFx (dinitro-Flexizyme). (b) Schematic representation of peptide cyclization *via* the spontaneous intramolecular reaction between Cab and Cys side chains.

significantly simplifying the process of designing cyclic peptides. Moreover, when a template DNA library containing fully or partially randomized sequences in the open-reading frame is prepared, a peptide library consisting of billions to trillions of sequences can be synthesized. By means of phage display (*13*), for instance, peptide ligands against a target protein can be screened. Importantly, such a constrained cyclic structure is often crucial to obtain high affinity ligands to the target. A disadvantage of the ribosomal synthesis is that usable building blocks are generally limited to proteinogenic amino acids, and thus the cyclization format is restricted to only the disulfide bond. Unfortunately, the disulfide bond is reducible under physiological conditions, and thereby such a peptide often loses its biological activity upon linearization and also becomes susceptible to peptidases (*14*).

To overcome the above drawbacks of cyclic peptides formed by a disulfide bond, several methods were devised but their application had been limited to only chemical synthesis of such peptides until recently (*15*); however, three research teams independently reported methods applicable to peptides expressed in cell-free translation systems. The first method reported by Szostak and coworkers (*16*) uses α,α′-dibromo-*m*-xylene as a cross-linker to cyclize a peptide containing two Cys residues expressed in a reconstituted *E. coli* cell-free translation (PURE) system (*17*). This methodology originally developed by Meloen *et al.* (*18*) generates two thioether bonds between two Cys residues and the cross-linker agent, inserting five carbons (approximately 8 Å) between the Cys−Cys bond. The second method reported by Roberts and coworkers utilizes disuccinimidyl glutarate as a cross-linker (*19, 20*). In this work, rabbit reticulocyte lysate translation system was used to express desired peptides containing an N-terminal free amine of Met and a single Lys in the nascent peptide chain, and the above cross-linker agent ligates the N-terminal amine and the Lys ε-amine. This linkage also inserts five carbons but is significantly longer than the Cys−Cys bond length due to the additional length of the side chain of Lys (by more than 11 Å).

In contrast to the above two methods requiring cross-linker agents for the conversion from a linear peptide to the cyclic form, our group has recently devised a new method that does not require such a post-translational manipulation. In this method, an N^{α}-(2-chloroacetyl)-amino acid (ClAc-aa) charged onto *E. coli* initiator $tRNA^{fMet}_{CAU}$ is used to initiate the translation using a Met-withdrawn PURE system (called *w*PURE system), yielding a peptide containing ClAc-aa at the N-terminus and a single Cys in the nascent chain (*21*). The translated peptide is cyclized by nucleophilic attack of the Cys side chain to the α-carbon of ClAc group at the N-terminus, resulting in the formation of a nonreducible thioether bond. Notably, this "N-terminus-to-side-chain closure" chemistry takes place spontaneously and nearly quantitatively *in situ* in the translation mixture independent of the peptide ring size. This linkage is approximately 3 Å shorter than the parental cyclic peptide formed by Cys−Cys linkage, thus giving a more constrained structure.

Here we expand this methodology to an "inter-side-chain" cyclization using N^{γ}-(2-chloroacetyl)-α,γ-diaminobutylic acid (Cab) as a new family of nonproteinogenic amino acids (Figure 1, panel a). Incorporation of Cab followed by Cys into the nascent peptide chain enables us to spontaneously generate a cyclic peptide closed by a thioether bond between these two side chains (Figure 1, panel b). Distinct from the previous "N-terminus-to-side-chain" linkage, this method leaves N- and C-terminal sequences untouched, so that this linkage is a substitute of the Cys−Cys bond with an insertion of the acetylamide moiety (approximately 5 Å longer than that of Cys−Cys). Using this new method, we have synthesized an analogue of human urotensin II (hU-II), a potent vasoconstrictor peptide (*22, 23*), closed by a thioether bond and demonstrated its biological activity. Remarkably, this analogue exhibits high peptidase resistance, in contrast to the Cys−Cys-linked peptide highly susceptible to proteolysis. Thus, this represents a novel method for generating nonstandard cyclic peptides and provides a powerful means to develop drug-like peptides.

RESULTS AND DISCUSSION

Incorporation of Cab into a Linear Peptide and Its Spontaneous Cyclization. To incorporate Cab into the nascent peptide chain, we used two technologies. The first is flexizyme, a *de novo* ribozyme tRNA aminoacylation catalyst (*24*). This artificial enzyme is able to charge virtually any amino acid assisted by 3,5-dinitrobenzyl ester (DBE) leaving group onto any desired tRNA; a very flexible tool for these type of studies. The second is a so-called PURE system (*17*). Since this *E. coli* cell-free translation system is reconstituted from recombinant protein factors, ribosome, and essential organic and inorganic sources, we can withdraw desired protein factors and/or amino acids from the components. In this work, we prepared a PURE system in which Leu was withdrawn (see Methods), and this PURE system was referred to as the *w*PURE system.

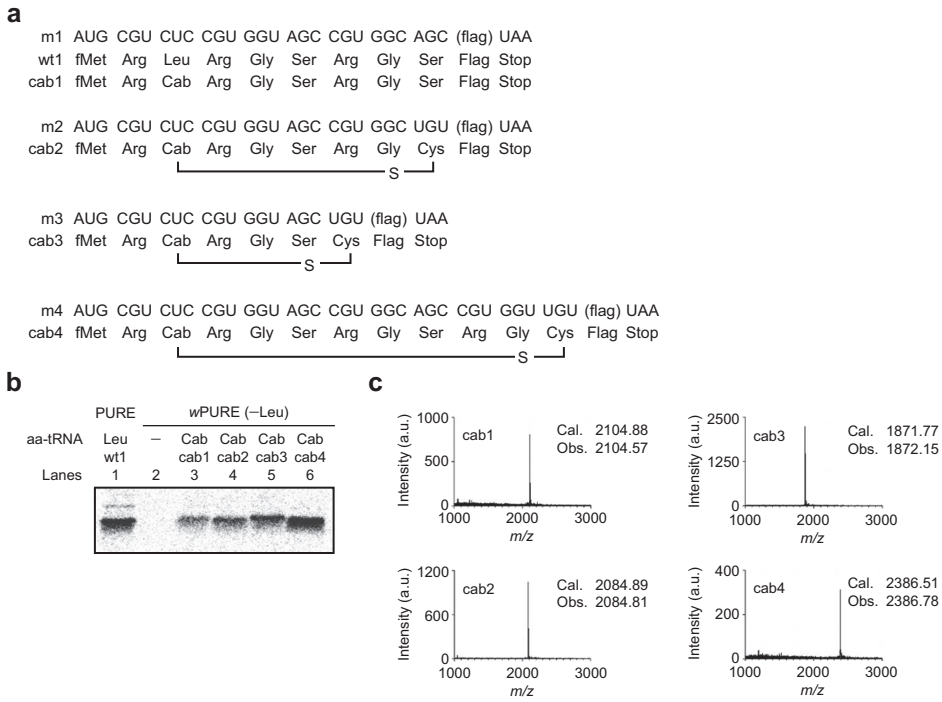

Figure 2. Incorporation of Cab into a peptide chain and its spontaneous cyclization. (a) Sequence of mRNAs (m1–4) and corresponding peptides (wt and cab1–4). Flag in parentheses indicates the RNA sequence encoding the Flag peptide. (b) Tricine SDS-PAGE analysis of the translated products labeled with [^{14}C]-Asp: lane 1, *in vitro* translation with the ordinary PURE system to yield wild type; lane 2, a negative control using *w*PURE system in the absence of aa-tRNA; lanes 3–6, incorporation of Cab into the respective peptides in the presence of Cab-tRNA$^{Asn\text{-}E1}_{GAG}$ prepared by the flexizyme system. In the *w*PURE system, Cab was coded by CUC codon. (c) MALDI-TOF MS spectra of the translated peptides containing Cab (cab1–4). The calculated molecular mass (Cal.) and observed molecular mass (Obs.) for singly charged species, $[M + H]^+$ are shown in the spectra.

We synthesized Cab-DBE and attempted to charge Cab onto a tRNA analogue, microhelix RNA, using flexizyme. This conventional analytical method was reliable to judge the flexizyme aminoacylation ability toward the chosen aminoacyl-DBE by separating the charged RNA from uncharged RNA using denaturing-acid-PAGE. As expected, flexizyme was able to charge Cab onto microhelix RNA with over a 50% yield (Supplementary Figure S1). We then prepared Cab-tRNA$^{Asn\text{-}E1}_{GAG}$ that suppresses Leu codon (CUC) by using flexizyme and Cab-DBE. This particular tRNA was chosen because of its orthogonal property against *E. coli* aaRS (*25*); even if Cab-free tRNA$^{Asn\text{-}E1}_{GAG}$ were generated by deacylation during the translation, it would remain uncharged with proteinogenic amino acids by aaRSs present in the *w*PURE system, thus repressing any undesirable background incorporations.

To evaluate the efficiency of single incorporation of Cab into a nascent peptide chain, we designed an open-reading frame (ORF) in mRNA that expresses a model peptide consisting of fMet-Arg-Leu-Arg-Gly Ser-Arg-Gly Ser followed by a Flag peptide (DYKDDDDK: D, [^{14}C]-Asp or Asp; Y, Tyr; K, Lys) as a wild-type peptide in the PURE system (Figure 2, panel a, wt1 and Figure 2, panel b, lane 1). In the *w*PURE system, the Leu codon (CUC) was suppressed by Cab-tRNA$^{Asn\text{-}E1}_{GAG}$, generating a Cab-containing peptide (Figure 2, panel a, cab1). Tricine SDS-PAGE analysis revealed that the peptide was visible only when Cab-tRNA$^{Asn\text{-}E1}_{GAG}$ was present in the *w*PURE system, giving an approximately 40% yield compared with that of wild type (Figure 2, panel b, lanes 2 and 3). To confirm the incorporation of Cab, cab1 peptide was analyzed by MALDI-TOF mass spectrometry, showing

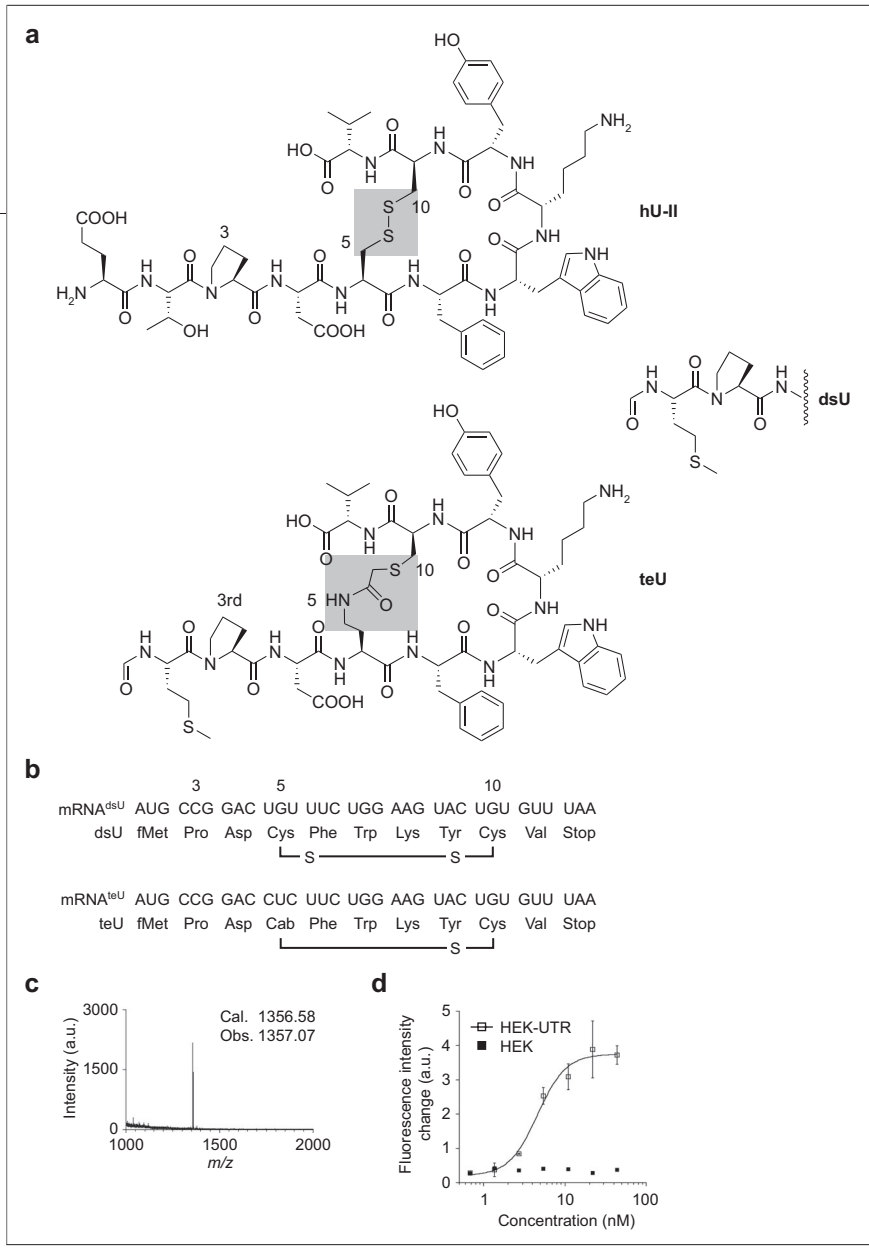

Figure 3. Expression of human urotensin II-like peptides. (a) Molecular structures of human urotensin II (hU-II), dsU, and teU. The inter-side-chain bond in each peptide is highlighted in a gray rectangle. (b) Sequences of mRNA templates encoding human urotensin II-like peptide, dsU (disulfide containing urotensin II-like peptide) and teU (thioether containing urotensin II-like peptide), and the corresponding peptides. (c) MALDI-TOF MS spectrum of teU. (d) Titration of calcium mobilization as a function of teU concentration in HEK-UTR and HEK cells. All experimental points were performed in triplicate.

that its observed molecular mass was consistent with the calculated mass (Figure 2, panel c, cab1). It should be noted that only a single peak corresponding to cab1 was observed, indicating that the 2-chloroacetyl group was intact during the translation, *i.e.* no side reaction occurred with ingredients such as mercaptoethanol and DTT (see supplementary note in the Supporting Information).

Although the 2-chloroacetyl group of Cab was intact in cab1 peptide, we expected that incorporation of a single Cys into the nascent peptide chain would result in selective intramolecular ring closure between Cab and Cys residues. To see whether the expected intramolecular reaction could take place, we designed an mRNA template and expressed a peptide (Figure 2, panel a, m2 and cab2) in which Ser in cab1 peptide was replaced with Cys. Upon the intramolecular cyclization of cab2, it was expected to form a 7-residue sized ring. We also designed two mRNA templates expressing shorter and longer peptides than the cab2 peptide (Figure 2, panel a, m3 and cab3; m4 and cab4), giving 5- and 10-residue ring sizes, respectively. All three peptides were expressed well, giving an expression level comparable to or even better than that of cab1 (Figure 2, panel b, lanes 4–6). MALDI-TOF analysis of the respective peptide showed a clean single product (Figure 2, panel c, cab2–cab4), and the observed molecular mass of the respective peptides was consistent with their expected molecular mass of the cyclic form closed by the thioether bond. These results indicated that the cyclization between Cab and Cys side chains took place spontaneously and nearly quantitatively *in situ* in the translation mixture. Importantly, the cyclization occurred independent of the ring sizes, proving this methodology of its general applicability.

Expression and Biological Activity of Human Urotensin II-Like Peptides. Human urotensin II (hU-II) is a potent vasoconstrictor that binds to a receptor, called the urotensin-receptor (UTR), that belongs to a family of G-protein coupled receptors (*22, 23*). hU-II consists of 11 amino acids and possesses a single disulfide bridge between two Cys residues in positions 5 and 10 (Figure 3, panel a, hU-II). In hU-II, not only endocyclic residues but also exocyclic residues play a critical role in exhibiting the biological activity *in vivo* (*26*). For instance, truncation of either Glu^1–Asp^4 at the N-terminus or Val^{11} at the C-terminus led to greater than 200-fold loss, and both truncations led to nearly completely loss of the vasoconstrictor activity. Considering the importance of the exocyclic residues of hU-II, our previous method "N-terminus-to-side-chain closure" is apparently not appli-

cable to this peptide. However, the "inter-side-chain closure" method developed in this work should be suited for this purpose. Here, the objective of our proof-of-concept study is 2-fold; we (1) demonstrate the synthesis of a hU-II analogue containing the nonreducible thioether bond (Figure 3, panel a, teU) and (2) show the retention of biological activity and its increased proteolytic stability under physiological conditions.

According to previous studies, the N-terminal residues of hU-II, Glu^1, and Thr^2 are not essential for the activity (*26, 27*). We therefore designed a template $mRNA^{dsU}$ expressing a hU-II-like peptide consisting of the minimal activity domain, where the translation would be initiated with fMet, elongated with Pro^3 and the rest of sequence, and eventually closed by the Cys^5-Cys^{10} disulfide bond (Figure 3, panels a and b, dsU). The dsU peptide expressed in PURE system was isolated by a conventional ultrafilteration and desalting purification protocol reported elsewhere (*21*). The MALDI-TOF analysis of dsU gave a clean single peak with the expected molecular mass (see Figure 4, panel b, pK–), suggesting that the peptide isolated by our simple protocol should be sufficient for biological assay. To estimate the peptide concentration, dsU was labeled with [^{14}C]-Asp and analyzed by a liquid scintillation counter calibrated against known [^{14}C]-Asp concentrations. We observed an approximately 1.3 μg/mL (=1.0 μM) expression level (data not shown).

To examine if dsU peptide retained the hU-II-like activity, we performed a calcium mobilization assay. HEK-293 cells stably expressing UTR, referred to as HEK-UTR, were preincubated with a fluorescent calcium indicator dye, Fluo-4 a.m., and then dsU was added to the medium. The fluorescent intensity change was monitored using a microtiter-plate reader. We titrated Ca^{2+} mobilization responses as a function of the dsU concentrations, giving a value of an effective concentration for the half-maximal response (EC_{50}) with 0.47 ± 0.11 nM (Table 1, Supplementary Figure S2a). Under the same conditions HEK-293 did not show any response to dsU even at greater than 2 orders of magnitude higher concentrations (data not shown), indicating that the response is strictly dependent upon the expression of UTR. Importantly, the observed value was consistent with that determined for a chemically synthesized authentic hU-II (EC_{50} = 0.55 ± 0.18 nM, Table 1, Supplementary Figure S2b). These results firmly established that our procedures involving the cell-free expression

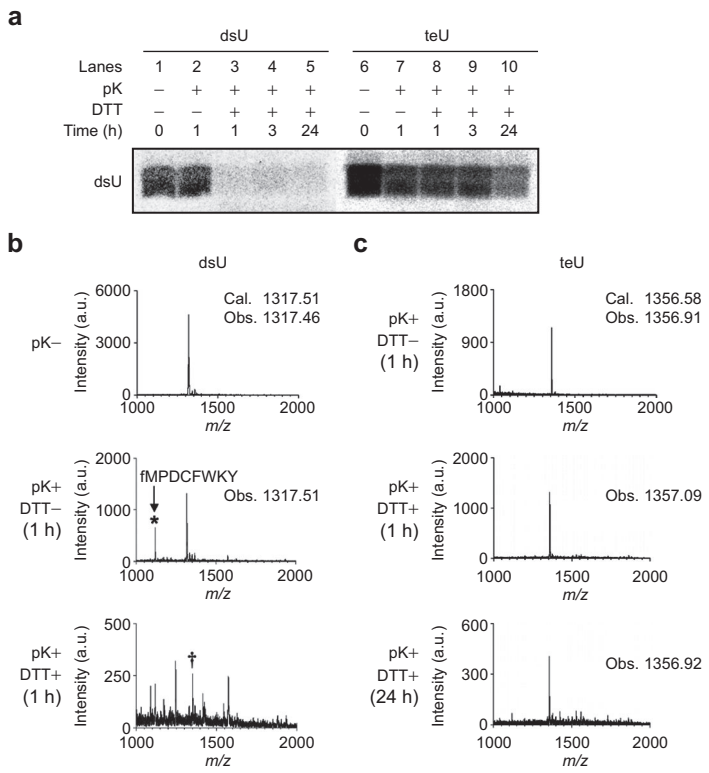

Figure 4. Analysis of peptide stability by proteinase K (pK). (a) Tricine SDS-PAGE analysis of dsU and teU incubated with pK. Peptides were labeled with [^{14}C]-Val and detected by autoradiography. dsU and teU were analyzed without pK (lanes 1 and 6) or with pK (lane 2–5 and 7–10), in the absence of DTT (lanes 2 and 7) or presence of DTT (lane 3–5 and 8–10) for indicated time periods. Note that the intensity of the teU band in lane 10 slightly decreased after 24 h, and this phenomenon was observed repeatedly in triplicate experiments. This could be attributed to a fraction of peptide being stuck to the tube surface and difficult to elute by buffer wash; alternatively, a minor degradation of teU could occur by the pK incubation although such fragments were not observed in the following MALDI-TOF analysis. (b, c) MALDI-TOF MS spectra of dsU and teU incubated with pK. In the absence of pK, both dsU (pK–) and teU (Figure 3, panel c) showed a single peak corresponding to the intact peptide. dsU and teU were then incubated with pK in the absence of DTT or presence of DTT for 1 h (pK+/DTT– 1 h, and pK+/DTT+ 1 h). In the spectrum of dsU without DTT, an additional peak (*) was observed, which corresponds to the hydrolyzed peptide between Tyr^9 and Cys^{10} (Cal. = 1117.45, Obs. = 1117. 45). In the spectrum of dsU with DTT, we could not detect any peak corresponding to the intact peptide (the peak corresponding to † near 1351 Da was a noise peak since the ms did not match to the intact dsU peak). teU was further incubated with pK in the presence of DTT for 24 h (pK+/DTT+ 24 h), showing the intact peptide peak.

of the peptide, purification, concentration estimation, and biological assay are reliable to evaluate hU-II-like peptides.

We then prepared a template $mRNA^{teU}$ (Figure 3, panel b), where the Leu codon was suppressed with

TABLE 1. Calcium mobilization assay in HEK-UTR cells

Name	Sequence	EC_{50} (nM)[a]
hU-II	⌐S—S¬ ETPDCFWKYCV	0.55 ± 0.18
dsU	⌐S—S¬ fMPDCFWKYCV	0.47 ± 0.11
teU	⌐——S¬ fMPDCabFWKYCV	4.6 ± 0.58

[a] EC_{50} indicates the effective concentration that gives the half-maximal response. The standard deviation was determined by experiments in triplicate.

Cab-tRNA$^{Asn\text{-}E1}_{GAG}$ giving the substitution of Cys^5 in dsU with Cab^5 (Figure 3, panels a and b, teU). Indeed, the translation of this template in *w*PURE system in the presence of Cab-tRNA$^{Asn\text{-}E1}_{GAG}$ expressed teU in a comparable yield to the dsU in a 1.2 μg/mL expression level (data not shown), and MALDI-TOF analysis of the product purified by our protocol showed a clean single product (Figure 3, panel c). Most importantly, the observed molecular mass of the peptide was matched with the expected molecular mass of the cyclic form closed by the thioether bond, indicating that the cyclization between Cab^5 and Cys^{10} side chains took place spontaneously and nearly quantitatively.

Having the cyclic teU in hand, we tested its ability for the induction of Ca^{2+} mobilization using HEK-UTR and HEK-293 cells under the same titration conditions performed for hU-II and dsU. HEK-UTR exhibited a Ca^{2+} mobilization response as a function of concentration of teU, and EC_{50} value was determined to be 4.6 ± 0.58 nM (Figure 3, panel d). In contrast, HEK-293 did not respond to teU at all, indicating that the response was clearly UTR-dependent. The EC_{50} value of teU was, however, approximately 10-fold higher than that for dsU, suggesting that the additional atomic length of the acetylamide group introduced to the linkage somewhat perturbed the original cyclic constrain. Yet it was remarkable that teU was able to induce Ca^{2+} mobilization at a low concentration (nM). Thus, our next question was whether teU possesses improved proteolytic stability.

Peptidase Resistance of teU. Considering the clinical use of peptides, their stability against peptidases (proteases) existing *in vivo* is crucial. Our goal was to show that the nonreducible thioether linkage implanted into the hU-II-like-peptide, teU, conferred peptidase resistance. Before testing this, we first treated three peptides with human plasma (HP) to see how stable the synthetic hU-II and *in vitro* translated dsU and teU were against peptidases. After incubation for 24 h with HP, hU-II was digested into two peptide fragments observed in MALDI-TOF; the cleavage occurred at two sites, between the first and second residues and between the third and forth residues (Supplementary Figure S3a). Interestingly, when dsU was treated with HP for 1 h or even 24 h, it exhibited notable stability for HP digestion (Supplementary Figure S3b). Likewise, teU also exhibited strong resistance against HP (Supplementary Figure S4a). Because the obvious difference of hU-II from dsU/teU was the absence or presence of a formyl group at the N-terminus, we hypothesized that the N-formy group of dsU/teU protected against peptidases in HP. Indeed, when nonformylated forms of deU and teU were prepared by translation, both peptides became susceptible to HP digestion (Supplementary Figures S3c and S4b). We concluded that the N-terminal formylation granted HP resistance.

Proteinase K (pK) is a nonspecific and highly active protease that can cleave various sequences of peptides. We considered that treatment of disulfide-bridged cyclic peptides with pK under reducing conditions would mimic extreme physiological situations. When dsU was treated with pK in the absence of DTT for 1 h, most dsU seemed intact as analyzed by tricine-PAGE radioautoradiography (Figure 4, panel a, lane 2), but MALDI-TOF analysis of this peptide showed a fragment corresponding to the C-terminal digestion at Tyr^9 and Cys^{10} residues (Figure 4, panel b, pK+/DTT–). Upon treatment of dsU with pK in the presence of DTT, the full-length peptide band completely disappeared after 1 h (Figure 4, panel a, lanes 3–5). This was consistent with the MALDI-TOF data where no corresponding full-length peak was observed (Figure 4, panel b, pK+/DTT+). These results clearly imply that the reduction of disulfide bond in dsU significantly increases pK susceptibility.

Substitution of the disulfide bond with the Cab^{5}–Cys^{10} thioether bond made a dramatic change in the pK susceptibility under reducing conditions. When teU was treated with pK, no degradation was detected under not only nonreducing conditions but also reducing conditions for 1 or 3 h (Figure 4, panel a, lanes 7–9). The band corresponding to the full-length peptide could be also observed even after 24 h, showing a remarkable pK-resistance (Figure 4, panel a, lane 10). This observation also well agreed with the observation by their MALDI-TOF analysis where the intact peak was cleanly observed after 1 and 24 h (Figure 4, panel c, pK+/DTT–, and pK+/DTT+ 1 or 24 h). Thus, the thioether bond implanted into teU along with the N-terminal formyl group conferred peptidase-resistance under reduced conditions.

CONCLUSION

We have demonstrated that a new member of nonproteinogenic amino acids containing the 2-chloroacetyl group in its side chain, named Cab, can be efficiently incorporated into a designated site in a peptide assigned by the genetic code reprogramming using the combination of flexizyme and *w*PURE systems. This unique chemical group on the side chain selectively reacts with the sulfhydryl group of Cys in the peptide chain in an intramolecular fashion, giving an inter-side-chain thioether bond. Remarkably, this chemistry takes place spontaneously *in situ* in the translation mixture independent of the ring sizes. We have applied this platform technology to the synthesis of a human urotensin II (hU-II) analogue closed by a nonreducible Cab-Cys thioether bond that replaces the Cys–Cys disulfide bond in hU-II. This substitution in this peptide, called teU, results in a mild reduction of its calcium mobilization activity but yet it exhibits the effective concentration for the half-maximal response (EC_{50}) with less than 5 nM. Most significantly, teU is highly peptidase-resistant against human plasma and proteinase K under reduced conditions in contrast to the parental hU-II (as well as dsU), which is susceptible to proteolysis.

Our data suggest that the cyclic peptide structure closed by the inter-side-chain thioether bond can be a generic scaffold that exhibits peptidase resistance. Also, this strategy is generally applicable to the synthesis of cyclic peptides independent of the ring sizes. These two features grant us a new opportunity to construct a new type of peptide library in the mRNA-encoding format. Particularly, the simplicity and reliability of our cyclization strategy enable us to readily couple this system with an appropriate *in vitro* display system, such as ribosome (*28*) or mRNA display (*29, 30*), to screen peptide aptamers from a large diverse library. Moreover, the expression level observed in our study (>1 μg/mL) also allows us to perform parallel synthesis of a focused cyclic peptide library for cell-based screenings. We expect these approaches to accelerate the discovery of novel peptidic drugs against various therapeutic targets.

METHODS

Translation of Wild-Type and Cab-Containing Peptides. The PURE system was prepared as described previously (*31*), which was reconstituted with ribosome, 20 aaRSs, protein factors, necessary organic and inorganic components including 1 mM DTT (note that an unknown concentration of mercaptoethanol would be also present due to carrying-over from the purification processes of aaRSs and protein factors), and 20 proteinogenic amino acids (0.2 mM each; for tricine SDS-PAGE analysis, 50 μM [^{14}C]-Asp was used in place of Asp), and all wild-type peptides were translated using this PURE system in the presence of a 40 nM DNA template (mDNA^{m1} and mDNA^{dsU}, see Supplementary Table S2) at 37 °C for 1 h at a scale of 5 μL total volume. *w*PURE system was reconstituted with the same components as the PURE system except that only necessary proteinogenic amino acids (0.2 mM each) and Cab-tRNA$^{Asn\text{-}E1}_{GAG}$ (50 μM) were added to the mixture depending upon the peptide kinds stated below: To express cab1 peptide, *w*PURE system in the presence of 40 nM mDNA^{m1}, 0.2 mM each of Met, Arg, Gly, Ser, Asp (this was replaced with 50 μM [^{14}C]-Asp for tricine SDS-PAGE analysis), Tyr, and Lys was used. To express cab2–cab4, the above *w*PURE system plus 0.2 mM Cys was used in the presence of 40 nM of the corresponding DNA template (mDNA^{m2-m4}). To express teU, *w*PURE system containing 0.2 mM each of Met, Pro, Asp, Phe, Trp, Lys, Tyr, Cys, and Val was used in the presence of 40 nM mDNA^{teU}. For tricine SDS-PAGE analysis, 2.5 μL of reaction mixture was taken and mixed with 2.5 μL of loading buffer (0.9 M pH 8.5 Tris-HCl, 30% glycerol, and 8% SDS) and analyzed by 15% tricine SDS-PAGE.

Mass Spectra Measurements of Peptides. For the mass analysis, peptides were purified by two different protocols. For FLAG-containing peptides (cab1–cab4), 5 μL of the reaction mixture was mixed with 5 μL of 2 × TBS (100 mM pH 8.0 Tris-HCl, 300 mM NaCl) and incubated in prewashed FLAG-M2 agarose (Sigma) for 1 h. The resin was then washed with 10 μL of TBS (50 mM pH 8.0 Tris-HCl, 150 mM NaCl) once, and the immobilized peptide was eluted with 2 μL of 0.2% TFA. The resulting peptide was desalted by C18 micro ZipTip (Millipore), and eluted with 1 μL of a 50% acetonitrile, 0.1% TFA solution saturated with the matrix (*R*)-cyano-4-hydroxycinnamic acid. The peptide lacking FLAG (dsU or teU) was purified with Microcon YM-10 centrifugal filter devices (Millipore). After the translation, 5 μL of the reaction mixture was diluted with 45 μL of water and added into the sample reservoir that was prewashed with water. The

reservoir was put into a vial, and centrifuged at 14000*g* for 20 min. The flow-through was collected and acidified with 1% TFA, followed by the ZipTip purification described above. Mass measurements were performed using MALDI-TOF (autoflex TOF/TOF, BRUKER).

Cell Culture and Transfection. HEK293 cells were grown in Minimum Essential Medium Eagle supplemented with 10% FCS, 100 units/mL of penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich) at 37 °C in a 5% CO_2 atmosphere. For the preparation of the cell line which stably expresses the U-II receptor, the cells were transfected with the human U-II-receptor cDNAs (UMR cDNA Resource Center, www.cdna.org) using the Effectene transfection reagent (QIAGEN) according to the manufacturer's protocol. Transfected cells were selected with G418, and a clonal cell line, designated as HEK-UTR, was obtained.

Calcium Mobilization Assay. After the translation, 5 μL of the reaction mixture was diluted with an equal amount of 1% TFA and purified with ZipTip. Peptides were eluted with 4 μL of 50% acetonitrile, 0.1% TFA solution. The eluent was concentrated by MV-100 Micro Vac (TOMY) for 10 min with heat and dissolved in 10 μL of assay buffer (Hanks Balanced Salts Solution (HBSS), 10 mM HEPES, 200 μM of $CaCl_2$, 0.1% BSA, and 2.5 mM probenecid). To determine the peptide concentration, 5 μL of translated peptides labeled with [^{14}C]-Asp were purified by ZipTIp as above, and the eluent was mixed with 10 mL of a liquid scintillation cocktail (Ultima Gold, Perkin-Elmer) and analyzed by a liquid scintillation counter (Wallac 1409, Wallac). The standard curve was created with 1 μL of various concentrations of monomer [^{14}C]-Asp. For the calcium mobilization assay, 2×10^5 HEK-UTR cells were seeded into poly(D-lysine)-coated 96-well black-wall, clear-bottom microtiter plates (Becton Dickinson) 12–16 h before the assay. Cells were loaded with 1 μM of the fluorescent calcium indicator Fluo-4 a.m. (Dojindo) in assay buffer for 1 h at 37 °C, 5% CO_2. After the incubation, cells were washed three times with the assay buffer and further incubated with 90 μL of the assay buffer for 10 min at 37 °C. The plate was then transferred to a microplate reader (Varioskan, Thermo Scientific) and the baseline fluorescence was measured for the first 10 s at a 2-s interval. Then 10 μL of the peptide solution with various concentrations was injected manually with a 10 μL scale microsyringe and the observed fluorescence was recorded for 2 min. The Ca^{2+} response was determined as a subtraction of maximum fluorescence intensity and average fluorescence intensity of the first 10 s.

Proteinase K Protection Assay. For the proteinase K (pK) protection assay, dsU and teU were translated in *w*PURE system containing 50 μM of [^{14}C]-Val, and 0.2 mM each of Met, Pro, Asp, Phe, Trp, Lys, Tyr, and Cys in the presence of 40 nM $mDNA^{dsU}$ or $mDNA^{teU}$ and 50 μM of Cab-$tRNA^{Asn\text{-}E1}_{GAG}$ only for teU synthesis. The respective peptide was purified by ZipTip as described in the calcium mobilization assay section. Eluent was concentrated in vacuo and dissolved in 2.5 μL of pK buffer (10 mM Tri-HCl buffer pH 7.5, 10 mM EDTA, 5 μg/mL pK) in the presence or absence of 1 mM DTT. The peptide was incubated at 37 °C for indicated time periods, and the peptidase digestion was stopped by heating the sample tube at 95 °C for 5 min. Samples were then analyzed by 15% Tricine-SDS PAGE. For MALDI-TOF MS analysis, translated peptide was first purified with Microcon, and flow-through was collected, concentrated and then dissolved in 2.5 μL of pK buffer with or without 1 mM DTT, followed by incubation at 37 °C for indicated time periods. Reaction was stopped by heating the sample tube at 95 °C for 5 min and analyzed by MALDI-TOF MS as described above.

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Supporting Information Available: This material is available free of charge *via* the Internet.

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