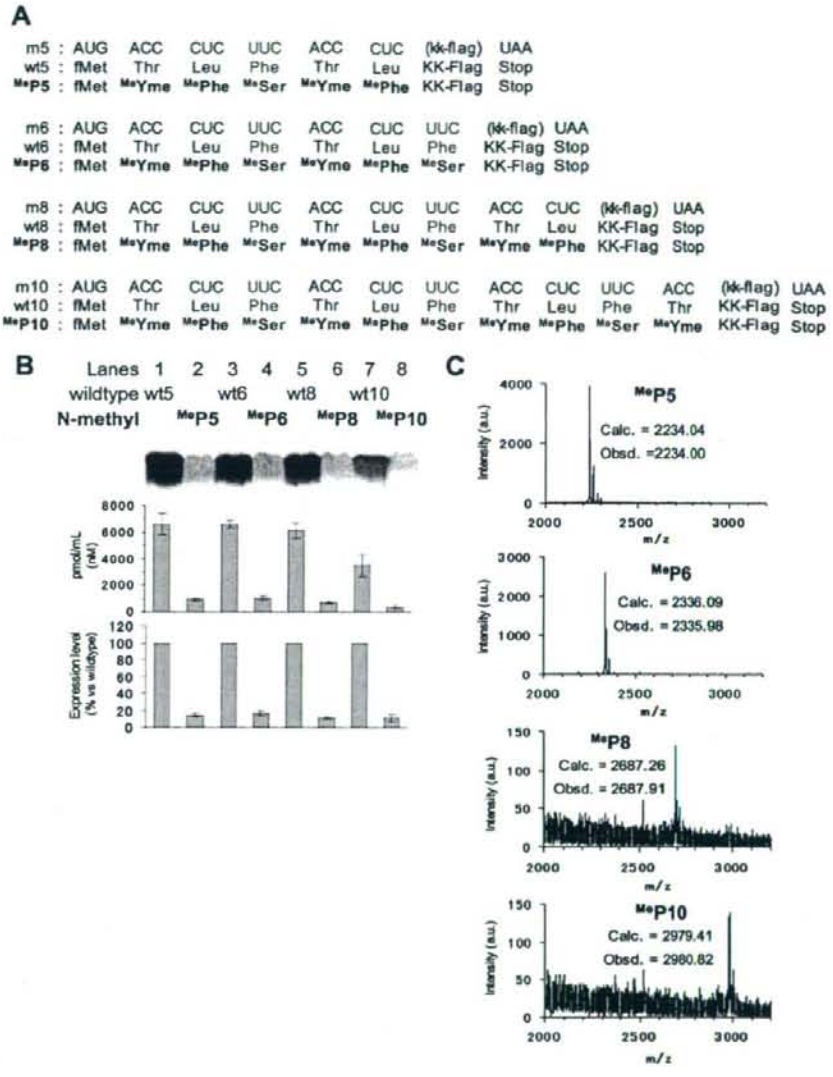

Figure 4. Expression of Longer N-Methyl-Peptides

(A) Sequences of mRNA templates (m5–10), control wild-type peptides (wt5–10), and peptides containing five, six, eight, or ten consecutive N-methyl-peptide bonds (^{Me}P5–10).

(B) Tricine-SDS-PAGE analysis of control wild-type peptides and N-methyl-peptides expressed from the respective mRNA. The peptides were labeled with [^{14}C]-Asp and detected by autoradiography. The wild-type peptides (lanes with odd numbers) were expressed in the ordinary PURE system, while N-methyl-peptides (lanes with even numbers) were expressed in the *w*PURE system containing ^{Me}Tyr-tRNA$^{Asn\text{-}E1}_{GGU}$, ^{Me}Phe-tRNA$^{Asn\text{-}E1}_{GAG}$, and ^{Me}Ser-tRNA$^{Asn\text{-}E1}_{GAA}$. Absolute expression level of each peptide based on its observed radioisotope counts is shown in the upper graph, and relative expression level of each N-methyl-peptide against the corresponding wild-type peptide is shown in the lower graph. Each expression level was determined by a mean score of triplicates.

(C) MALDI-TOF-MS spectra of ^{Me}P5–10. The calculated mass (Calcd.) and observed mass (Obsd.) are shown in each spectrum.

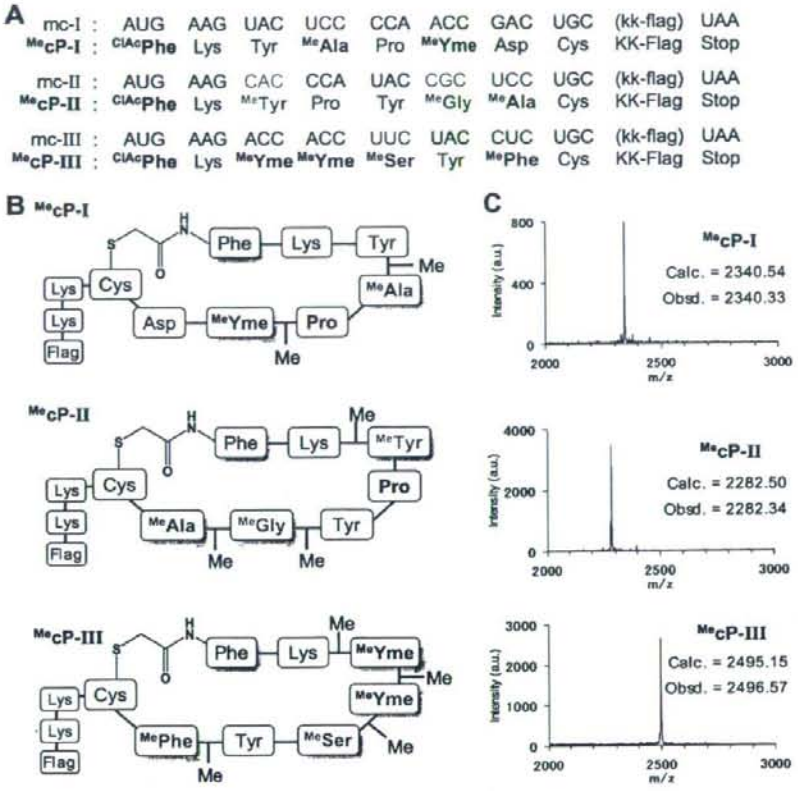

Figure 5. Synthesis of Cyclic N-Methyl-Peptides Closed by a Thioether Bond

(A) Sequences of mRNA templates (mc-I–III) and the precursor N-methyl-peptides (the linear version of ^{Me}cP-I–III). The codons and amino acids are colored according to reprogrammed genetic code table in Figure 1C.

(B) Structures of cyclic N-methyl-peptides. The locations of N-methyl groups on the peptide bond are shown in yellow.

(C) MALDI-TOF-MS spectra of cyclized ^{Me}cP-I–III. The calculated mass (Calcd.) and observed mass (Obsd.) are shown in each spectrum.

however, that in such a *w*PURE system, the incorporations of ^{Me}aa often suffered from contaminations caused by the competing incorporations of the proteinogenic amino acids to the assigned sites (data not shown). This was because that even though proteinogenic amino acids were supposedly depleted from the translation components, the recombinant translation factors and ribosome were often contaminated with small amounts of proteinogenic amino acids; therefore, they were charged onto the endogenous cognate tRNAs and readily competed out ^{Me}aa for the incorporation at the reprogrammed codon sites. Thus, it was critical to remove both aaRSs and proteinogenic amino acids from the translation components in order to repress the background incorporations of proteinogenic amino acids into the peptide. Moreover, we used orthogonal tRNAs as ^{Me}aa carriers aiming at eliminating potential mischarging events with proteinogenic amino acids catalyzed by aaRSs left in the *w*PURE system. Combining all of these controlled elements in the *w*PURE system, we were able to wipe out undesirable backgrounds and thus succeeded in synthesizing N-methyl-peptides as programmed by the mRNA.

Another key technology used in our proof-of-concept study is the flexizyme system. This system enables us to readily prepare a wide variety of ^{Me}aa-tRNAs with high purities, i.e., no contamination of the competing aa-tRNAs, so that we can ensure ^{Me}aa reassignments to the reprogrammed codons and thus determine the accessible ^{Me}aa in a quantitative manner. This technology has given us advantage over Merryman's method involving reductive alkylations on the precharged aa-tRNAs, where certain amounts of competing aa-tRNAs could remain unreacted and therefore contaminate the ^{Me}aa-tRNAs (Merryman and Green, 2004). As was discussed earlier, this might have led them to a different conclusion from ours on the accessible ^{Me}aa.

In a similar context, it would be difficult to perform ^{Me}aa assignments by the Szostak's method, where endogenous aaRSs or their mutants are used to mischarge nonproteinogenic amino acids onto endogenous tRNAs (Hartman et al., 2006; Josephson et al., 2005; Seebeck and Szostak, 2006). The major advantage of the Szostak's method is to in situ generate the mischarged aa-tRNAs and thus express the nonnatural peptides by simply adding nonproteinogenic amino acids to the *w*PURE system that lacks the corresponding proteinogenic amino acids. This system has been proven to be well suited for the incorporation of "highly efficient" nonproteinogenic amino acids into the target peptide, generally those structurally similar to proteinogenic amino acids that can readily compete out the background incorporations of amino acids originated by their contaminations (Seebeck and Szostak, 2006). Szostak et al. have also surveyed the aminoacylation ability of aaRSs toward ^{Me}aa, suggesting that ^{Me}Leu, ^{Me}Asp, ^{Me}Lys, ^{Me}His, and ^{Me}Trp could be charged onto the cognate tRNAs (Hartman et al., 2006). Unfortunately, among them only ^{Me}His and ^{Me}Trp were the "accessible" ^{Me}aa according to our experimental results shown in Figure 2C. As was discussed earlier, because of the intrinsic poor incorporation efficiency of ^{Me}aa, such background incorporations would give impure products containing certain or occasionally significant amounts of partially non-N-methylated peptides. In fact, by means of in situ aaRS's aminoacylation we found that ^{Me}His could be incorporated to the designated site of the same model peptide with competing incorporation of a smaller amount of His, whereas no incorporation of ^{Me}Trp, i.e., only incorporation of the competing Trp, was observed (T.K. and H.S., unpublished data).

Under the optimal conditions for the *w*PURE and flexizyme systems, we performed the mRNA-dependent synthesis of N-methyl-peptides containing more than four successive ^{Me}aa utilizing three different kinds of ^{Me}aa (Figures 3A and 4A). We monitored the expression level of each N-methyl-peptide by tricine-SDS-PAGE compared with the wild-type expression level based on their radioisotope intensities originating from the incorporation of [^{14}C]-Asp in the Flag peptide (Figures 3B and 4B). Using this strategy, we were able to directly compare the expression levels of the N-methyl-peptides in a quantitative manner.

Because we chose only accessible ^{Me}aa for the polymerization, we expected that the expression level would not be drastically reduced compared to the single incorporation. However, the expression of a tetra-N-methyl-peptide (^{Me}P4-I) gave only 20% of the wild-type expression level (Figure 3B, lanes 1 versus 5). Moreover, when longer N-methyl-peptides (from 5-mer to 10-mer) were expressed, their relative expression levels did not significantly change (Figure 4B). We wondered why the observed relative expression level of the tetra-N-methyl-peptides drastically dropped from that observed for the mono-N-methyl-peptide. To investigate this, we expressed mono-, di-, tri-, and tetra-N-methyl-peptides (Figure S3) (^{Me}P1-II, ^{Me}P2-II, ^{Me}P3-II, and ^{Me}P4-II) and compared their expression levels with those of the corresponding wild-type peptides (wt1-II, wt2-II, wt3-II, and wt4-II). Interestingly, the absolute expression levels of wt3-II and wt4-II were approximately three and four times higher, respectively, than that of wt1-II. On the other hand, the absolute expression levels of all N-methyl-peptides remained nearly the same. Thus, it became apparent that the relative expression levels of ^{Me}P3-II and ^{Me}P4-II to wt3-II and wt4-II appeared lower because the expression levels of wt3-II and wt4-II increased from wt1-II, whereas the absolute expression level of all N-methyl-peptides including 5- to 10-mers did not change significantly. Most importantly, MALDI-TOF analysis of the respective N-methyl-peptide gave the corresponding single peak with the correct molecular mass regardless of the length of the N-methyl-peptide. These findings led us to attempt the synthesis of cyclic-N-methyl-peptides.

In this application, we combined our technology for the ^{Me}aa incorporation into peptides with the method previously developed for the initiation reprogramming with *N*^{a}-ClAc-aa where a linear peptide containing Cys is expressed to afford the corresponding cyclic-peptide closed by a thioether bond between the N-terminus and the sulfhydryl group of the Cys. Because the thioether bond is nonreducible and thereby physiologically stable, it has been implanted into some therapeutic peptides in order to make them compatible for in vivo examination. Although such cyclic peptides were previously generated by only solid-phase chemistry, this method has enabled us to synthesize such cyclic peptides by means of the translation apparatus. Thus, the combination of the above two methods has offered us a platform technology for the synthesis of cyclic N-methyl-peptides by designing the corresponding mRNA templates. Remarkably, the expressed full-length cyclic N-methyl-peptides were highly pure in all three examples presented in this work. Thus, this technology is well suited for the preparation of libraries of cyclic N-methyl-peptides. Particularly, when this is coupled with in vitro display systems, such as ribosome (Hanes and Pluckthun, 1997) or mRNA displays (Nemoto et al., 1997; Roberts and Szostak, 1997), it becomes a very powerful means of screening large libraries and selecting in vivo-compatible cyclic N-methyl-peptides against therapeutic targets. Such experimental examples are shortly up coming from our laboratory.

SIGNIFICANCE

Reprogramming the genetic code allows for the simultaneous incorporation of multiple amino acid monomers with a wide variety of side chains into peptides through the ribosomal translation reaction. In this study, we achieved the mRNA-directed synthesis of various sequences and lengths of peptides containing multiple N-methylated amino acid residues upon reprogramming the genetic code by using the *w*PURE and flexizyme systems. Our demonstration shows the potential of this technology that leads us to a new strategy to construct libraries of linear and cyclic N-methyl-peptides simply by designing mRNA sequence libraries. Upon combining with appropriate screening strategies, this technology will yield a novel class of therapeutic peptides with protease resistance and cell permeability.

EXPERIMENTAL PROCEDURES

Materials and Synthesis of *N*^{a}-Methyl-Amino Acid Substrates

All *N*^{a}-methylated amino acids except *N*^{a}-methyl-L-cysteine and *N*^{a}-methyl-L-methionine were purchased from Watanabe Chemical, Japan. *N*^{a}-methyl-L-cysteine was synthesized by the procedure reported before from L-cysteine as a starting material (Park and Kim, 2002). *N*^{a}-tBoc-*N*^{a}-methyl-L-methionine was synthesized by the procedure reported before from *N*^{a}-tBoc-L-methionine as a starting material (Lee et al., 1990). *N*^{a}-methylated amino acids with the

aromatic side-chain (^{Me}Phe, ^{Me}Tyr, ^{Me}Trp, ^{Me}Fni, and ^{Me}Yme) were converted to cyanomethyl esters (CMEs), and the others were converted to 3,5-dinitrobenzyl esters (DBEs) by the procedure reported elsewhere (Murakami et al., 2006).

Synthesis of *N*"-Methyl-Aminoacyl-tRNA

Aminoacylation reactions were generally performed as follows: 12.5 μl of 20 μM tRNA in 0.2 M HEPES-KOH (pH 7.5) was heated at 95°C for 1 min and cooled to room temperature over 5 min. Five microliters of 0.1 M $MgCl_2$ and 2.5 μl of 0.1 mM dFx or 5 μl of 3 M $MgCl_2$ and 2.5 μl of 0.1 mM eFx were added to the solution. Five microliters of 25 mM *N*"-methylated amino acid substrate (DBE or CME) in DMSO was then added to the mixture and incubated on ice for 2–24 hr. For ^{Me}Thr, ^{Me}Tyr, ^{Me}Fni, and ^{Me}Yme, 5 μl of 200 mM substrate was used. The acylation reaction was quenched by addition of 150 μl of 0.6 M sodium acetate (pH 5.0), and the tRNA was recovered by ethanol precipitation. The pellet was rinsed twice with 70% ethanol with 0.1 M sodium acetate (pH 5.0) and once with 70% ethanol.

Ribosomal Synthesis of Linear N-Methylated Peptides and Wild-Type Peptides

The following conditions were used for the peptide synthesis without [^{14}C]-labeling. For the synthesis of N-methylated peptides (^{Me}P4-I, ^{Me}P4-II, ^{Me}P4-III, ^{Me}P4-IV, ^{Me}P5, ^{Me}P6, ^{Me}P8, ^{Me}P10, ^{Me}P1-II, ^{Me}P2-II, and ^{Me}P3-II), translation reaction was carried out by using the *w*PURE system (see the Supplemental Data) in the presence of 40 nM DNA template (one of the following templates: m4-I, m4-II, m4-III, m4-IV, m5, m6, m8, m10, m1-II, m2-II, or m3-II), 0.1 mM of the respective ^{Me}aa-tRNA(s), and 0.2 mM each of Met, Asp, Tyr, and Lys. For the synthesis of wild-type peptides (wt4-I, wt5, wt6, wt8, wt10, wt1-II, wt2-II, wt3-II, and wt4-II), translation reaction was carried out by using the ordinary PURE system in the presence of 40 nM DNA template (one of the following templates: m4-I, m5, m6, m8, m10, m1-II, m2-II, m3-II, or m4-II) and 0.2 mM each of Met, Thr, Leu, Phe, Asp, Tyr, and Lys. The translation reactions were assembled on ice and started by incubating at 37°C for 3 hr. For the detection of the peptides using autoradiography, see the section of tricine-SDS-PAGE analysis.

Tricine-SDS-PAGE Analysis

For tricine-SDS-PAGE analysis, peptides were synthesized by using 50 μM [^{14}C]-Asp in the place of 0.2 mM Asp. The translation reaction (2.5 μl) was stopped by the addition of 2.5 μl loading buffer consisting of 0.9 M Tris-HCl (pH 8.5), 30% glycerol, and 8% SDS for loading to 15% tricine-SDS-PAGE. Separation gel contained 15% acrylamide (acrylamide: bisacrylamide = 19:1), 1 M Tris•HCl (pH 8.5), 0.1% SDS, and 13% Glycerol, while the stacking gel contained 4% acrylamide (acrylamide: bisacrylamide = 29:1), 0.75 M Tris•HCl (pH 8.5), 0.075% SDS. The anode running buffer was 200 mM Tris•HCl (pH 8.9), while the cathode running buffer contains 100 mM Tris, 100 mM tricine, 0.1% SDS (the resulting buffer was approximately pH 8.3). The products, labeled with [^{14}C]-Asp in the C terminus FLAG peptide, were quantified by autoradiography with an image analyzer, FLA-5100 (Fuji, Japan), and the specific activity of [^{14}C]-Asp.

Ribosomal Synthesis of Cyclic N-Methylated Peptides

For the synthesis of cyclic N-methylated peptide (^{Me}Pc1, ^{Me}Pc2, or ^{Me}Pc3), translation reaction was carried out by using the *w*PURE system with 40 nM DNA template (mc1, mc2, or mc3) in the presence of 0.1 mM *N*"-ClAc-Phe-$tRNA^{fMet}_{CAU}$, 0.1 mM of the respective ^{Me}aa-tRNAs, and 0.2 mM each of Met, Lys, Tyr, Pro, Asp, and Cys. The translation reactions were assembled on ice and started by incubation at 37°C for 3 hr.

MALDI-TOF-MS Analysis

The respective peptide was incubated in prewashed Flag-M2 agarose (Sigma) for an hour. After the resin was washed with TBS (50 mM Tris-HCl [pH 8.0], 150 mM NaCl), the peptide was eluted with 0.2% TFA. Eluted peptide was desalted with C18 zip tips (Millipore) and eluted with 50% acetonitrile, 0.1% TFA solution saturated with the matrix (*R*)-cyano-4-hydroxycinnamic acid. MALDI-TOF-MS analysis was performed by using Autoflex TOF/TOF (Bruker Daltonics) operated in the linear positive mode and externally calibrated with Substance P (1348.66 Da), Bombesin (1620.88 Da), ACTH clip 1–17 (2094.46 Da), and Somatostatin 28 (3149.61 Da) standards.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures for RNA synthesis, tRNA aminoacylation, and preparation of the translation systems and are available at http://www.chembiol.com/cgi/content/full/15/1/32/DC1/.

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ACS chemical biology

Reprogramming the Translation Initiation for the Synthesis of Physiologically Stable Cyclic Peptides

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ABSTRACT The initiation codon dictates that the translation initiation event exclusively begins with methionine. We report here a new technology to reprogram the initiation event, where various amino acids and those bearing N^{α}-acyl groups can be used as an initiator for peptide synthesis. The technology is built upon the concept of genetic code reprogramming, where methionine is depleted from the translation system and the initiation codon is reassigned to the desired amino acid. We have applied this technology to the synthesis of an antitumor cyclic peptide, G7−18NATE, closed by a physiologically stable bond, and it is also extended to the custom synthesis of its analogues with various ring sizes. Significantly, cyclization occurs spontaneously upon translation of the precursor linear peptides. To demonstrate the practicality of this methodology, we also prepared a small cyclic peptide library designated by 160 distinct mRNAs. Thus, this technology offers a new means to prepare a wide array of *in vivo* compatible cyclic peptide libraries for the discovery of peptidic drug candidates against various therapeutic targets.

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Polypeptide synthesis in prokaryotic translation is generally initiated with N^{α}-formyl methionine (fMet) (*1*). The initiation process involves methionylation of the initiator tRNA ($tRNA^{fMet}_{CAU}$) catalyzed by methionyl-tRNA synthetase (MetRS) followed by formylation of the α-amino group on Met by methionyl-tRNA formyltransferase (MTF); the resulting fMet-$tRNA^{fMet}_{CAU}$ subsequently binds to initiation factors (IFs) and the complex enters the peptidyl-tRNA site of the ribosome to initiate translation (*2*, *3*). Although strict governance by the above mechanism controls the initiation event starting with fMet at the N-terminus exclusively, two methods have been devised to circumvent this requirement (*4*–*7*).

The first method involves engineering of the initiator tRNA where the CAU anticodon is altered to a triplet that assigns another amino acid (*4*, *5*). Critical tRNA recognition elements in some aminoacyl-tRNA synthetases (aaRSs) reside in the anticodon interaction (*8*). Therefore, the transplantation of an appropriately chosen anticodon into $tRNA^{fMet}$ is able to deceive the recognition by aaRS, leading to a mischarging of the cognate amino acid onto the engineered initiator $tRNA^{fMet}_{XXX}$ (XXX indicates the implanted anticodon). When such a tRNA, together with a mRNA containing the countercodon to the tRNA anticodon, is overexpressed in *Escherichia coli* the translation starts with the mischarged N^{α}-formylated amino acid. Since not all aaRSs can be deceived by this type of tRNA engineering, only a limited number of aaRSs (Val, Phe, Gln, Ile, and Lys) were able to mischarge the cognate amino acids onto $tRNA^{fMet}_{XXX}$ and lead to initiation.

The second method also involves engineering of the initiator tRNA. The initiator tRNA anticodon is altered to CUA and the mRNA AUG codon is replaced with the UAG amber codon (*6*, *7*), in a fashion similar to the incorpo-

ration of nonproteinogenic amino acids during the elongation event (*9–11*). When such a tRNA is *chemically* aminoacylated through multistep synthesis (*12*) and subjected to *in vitro* translation, the designated amino acid initiates peptide expression. Unfortunately, this approach suffers from the contamination of polypeptides initiated by other amino acids since the deacylated $tRNA^{fMet}_{CUA}$ can be recharged with the amino acids by cognate endogenous aaRSs (*6, 7*). This method has thus far demonstrated the initiation of translation with only a limited number of amino acids (Met, Val, and Lys) tagged with fluorescent or biotin groups for the purpose of expressing N-end-labeled polypeptides *in vitro*.

Taken together, the currently available technologies do not allow for the synthesis of polypeptides with a well-controlled N-terminal modification, and it remains unclear whether more diverse types of amino acids including those with various N^{α}-acyl groups are tolerated in the initiation event. We here have used the concept of genetic code reprogramming to engineer the initiation event. This new technology allows us to expand the repertoire of usable α-amino acids and their N^{α}-acyl groups for initiation.

As an application, we have demonstrated the ribosomal synthesis of an antitumor cyclic peptide, G7–18NATE, closed by a physiologically stable thioether bond. Significantly, the cyclization occurs spontaneously upon the translation of its precursor linear peptide so that no additional manipulation is necessary after translation. Moreover, this technology is applicable to the synthesis of cyclic peptides with a wide range of ring sizes, and thus it offers a novel tool to construct mRNA-programmed cyclic peptide libraries for the discovery of peptidic drug candidates with *in vivo* compatibility.

RESULTS AND DISCUSSION

Genetic Code Reprogramming for the Initiation Event. Reprogramming the genetic code is a technique in which the codons assigned to proteinogenic amino acids are reassigned to nonproteinogenic amino acids (*13*). This technique has been utilized for the reprogramming of the elongation event where multiple nonproteinogenic amino acids have been successfully incorporated into the nascent peptide chain (*13–20*). In principle, it is also applicable to the alteration of initiation, but it has not been yet extensively used for such an aim.

To perform reprogramming of the translation initiation, we have employed two key systems to facilitate reprogramming of the initiation event. The first one is an *Escherichia coli* reconstituted cell-free translation system, referred to as the PURE system (*21*). By *withdrawing* certain amino acids from the translation system (referred to as *w*PURE), vacant codons can be created in the genetic table. Met is depleted to make the initiation codon vacant, and therefore an alternative amino acid (Xaa) can be reassigned to initiation (Figure 1, panel a). The second key system is a *de novo* tRNA acylation system consisting of an artificially evolved ribozyme, termed flexizyme (Figure 1, panel b) (*20, 22, 23*). Flexizyme allows for virtually any amino acid to be charged onto tRNA. Thus, we expect that by filling the vacant start codon in *w*PURE system with Xaa-$tRNA^{fMet}_{CAU}$ prepared with the flexizyme system, *i.e.*, through the integration of these two systems, the initiator Met can be readily reassigned to any noncanonical amino acid (Figure 1, panel c).

To test whether these two systems could be integrated to reprogram the initiation event, a 14-mer peptide bearing a Flag tag sequence (*24*) at the C-terminus was expressed in the *w*PURE system lacking Met in the absence or presence of Met-$tRNA^{fMet}_{CAU}$ prepared by the flexizyme system (Figure 2, panel a). As a positive control, the wild-type peptide was expressed in the normal PURE system containing the full sets of amino acids (Figure 2, panel b, lane 1). As a negative control, when only the *w*PURE system was used, initiation did not occur and thereby no peptide was produced (Figure 2, panel b, lane 2). In contrast, when Met-$tRNA^{fMet}_{CAU}$ prepared by the flexizyme system was included in the *w*PURE system, initiation proceeded and yielded the expected band with the same intensity and mobility as the wild type (Figure 2, panel b, lane 3 *vs* lane 1). Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis of the product gave the expected molecular mass of the peptide bearing fMet at its N-terminus (Figure 2, panel c, Met). Thus, Met-$tRNA^{fMet}_{CAU}$ synthesized by the flexizyme system was formylated by endogenous MTF and able to initiate translation as efficiently as the native initiation event in the normal PURE system. These results demonstrated that the concept of genetic code reprogramming would be applicable to engineering of the initiation event as well as the elongation event aiming to express peptides containing various amino acids at the N-terminus.

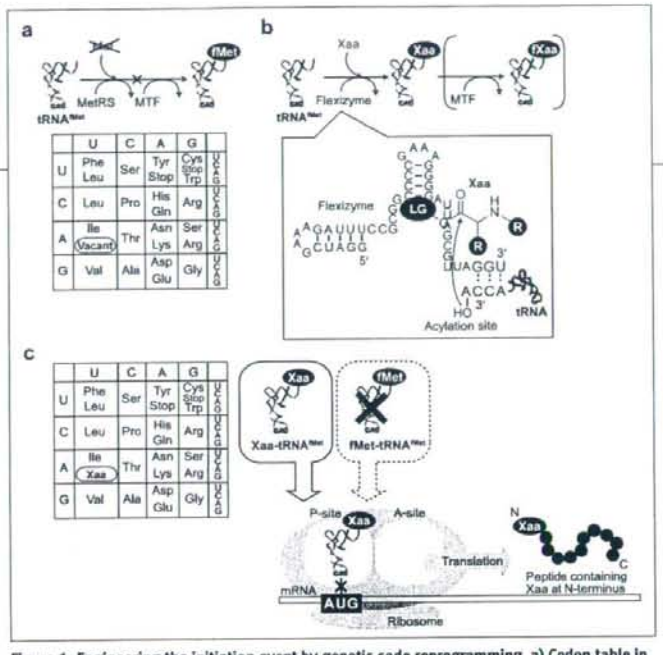

Figure 1. Engineering the initiation event by genetic code reprogramming. a) Codon table in the absence of Met. Upon removing Met from the translation mixture, $tRNA^{fMet}_{CAU}$ is no longer available for methionylation by MetRS, consequently making the start codon vacant, *i.e.*, *w*PURE system. b) Synthesis of Xaa-$tRNA^{fMet}_{CAU}$ by the flexizyme system. Xaa indicates various kinds of α-amino acid. The secondary structure of a representative flexizyme is shown in cyan letters. When Xaa-$tRNA^{fMet}_{CAU}$ is added to the translation apparatus, it can be formylated by endogenous MTF. c) Reassignment of the start codon from Met to Xaa. When Xaa-$tRNA^{fMet}_{CAU}$ prepared by the flexizyme system is added to the *w*PURE system, the translation is exclusively initiated with Xaa, expressing a peptide containing Xaa at the N-terminus.

Tolerance of Amino Acids in Initiation. We next tested initiation using each of the 19 proteinogenic amino acids by charging each amino acid to $tRNA^{fMet}_{CAU}$ with the flexizyme system. Tricine–SDS PAGE (soduim dodecyl sulfate polyacrylamide gel electrophoresis) analysis of the translation products showed that 11 amino acids out of 19 were able to initiate translation with greater than 50% efficiency relative to wild type (Figure 2, panel b, highlighted in orange). In addition, two amino acids exhibited the ability to initiate translation with approximately 30–50% efficiency relative to wild type (Figure 2, panel b, highlighted in pink). MALDI-TOF analysis of these translated peptides gave a single peak with the expected molecular mass of the N-terminal-formylated form initiated with the programmed amino acid (Figure 2, panel c, highlighted in the same color code as Figure 2, panel b). This indicates that the initiation occurs exclusively with the N^{α}-formylated amino acid as programmed by the flexizyme system.

The remaining six amino acids (Glu, Lys, Gly, Asp, Arg, and Pro) showed less than 30% translation efficiency compared to wild-type expression, with Arg and Pro producing yields in the single digits (Figure 2, panel b, highlighted in cyan). Despite their poor yields, the Flag-purified peptides initiated by Glu, Lys, Gly, and Asp gave the expected molecular mass of the formylated form, indicating that the full-length peptides were correctly initiated with the designated amino acids. Albeit with low efficiency, even Arg and Pro were able to initiate translation giving peaks corresponding to peptides initiated with programmed amino acid in the respective mass spectrum, although Arg gave a mixture of formylated and nonformylated peptides while Pro only produced the nonformylated peptide (Figure 2, panel c).

Interestingly, the majority of "good" initiators have hydrophobic side chains. On the other hand, six amino acids exhibited less than 30% efficiency in initiation (Figure 2, panel b, highlighted in cyan); among them, four have charged side chains (Lys, Glu, Asp, and Arg), one has no side chain (Gly), and the least efficient initiator Pro has the secondary amine. We assumed that inefficient initiation with these amino acids was caused by poor formylation of the α-amino group on the Xaa-$tRNA^{fMet}_{CAU}$ catalyzed by MTF. In the crystal structure of the complex between fMet-$tRNA^{fMet}_{CAU}$ and MTF, the side chain of Met fits into a binding pocket surrounded by hydrophobic amino acids (*25*). Presumably, this hydrophobic environment might dismiss the charged or hydrophilic side chains resulting in poor formylation on the α-amino group, while Pro, the only amino acid bearing the secondary amine, is likely enzymatically incompatible with MTF. Indeed, the two least efficient initiators, Arg and Pro, afforded nonformylated peptide in each mass spectrum of the product.

The above results indicated that the integration of the *w*PURE and flexizyme systems enabled us to readily reassign the AUG codon from Met to other amino acids and express the desired peptide initiated with the assigned amino acid. Although traces of undesirable peptides with slower or/and faster mobility were detected in the tricine-SDS PAGE analysis, these bands also appeared in the control experiment in the absence of aa-$tRNA^{fMet}_{CAU}$ (Figure 2, panel b, lane 2). This suggested that these bands appeared from aa-$tRNA^{fMet}_{CAU}$-independent expressions of the mRNA template, likely

due to in-frame mis-initiations. However, the expression levels of these peptides were the negligible background, and indeed we observed only a single desirable full-length product in the MALDI-TOF analysis. This was a sharp contrast to the previous method that suffered from contaminations of undesirable full-length peptides mis-initiated with undesignated amino acids.

We believe that the observed nearly exclusive expression of the desirable peptides has been achieved by two critical strategies we took for reprogramming the translation initiation event. First, depletion of Met in the *w*PURE system enabled us to suppress the background initiation reaction as low as possible. Second, the flexizyme system enabled us to use the natural initiator $tRNA^{fMet}_{CAU}$ as an aminoacyl-carrier for noncognate amino acids, and thereby we achieved a maximal efficiency for initiating the translation with each amino acid. By the combination of these two strategies, we were able to express the full-length peptide initiated with the designated noncanonical amino acid as a sole product.

Enhancing the Initiation Efficiency by Preacylation of α-Amino Group. As described above, an intriguing observation was made regarding the Arg-initiated peptide in which the N-terminus was not fully formylated (Figure 2, panel c, Arg). Likewise, the N-terminus of Pro-initiated peptide was not formylated at all (Figure 2, panel c, Pro). This result implies that these inefficient initiators are poor substrates for MTF (Figure 3, panel a). This observation led us to postulate that the efficiency of formylation on the α-amino group of Xaa-$tRNA^{fMet}_{CAU}$ by MTF might play an important role in determining the initiation efficiency, since the expression levels of these peptides were significantly lower than peptides initiated with other amino acids. RajBhandary and coworkers (*26*) have also proposed

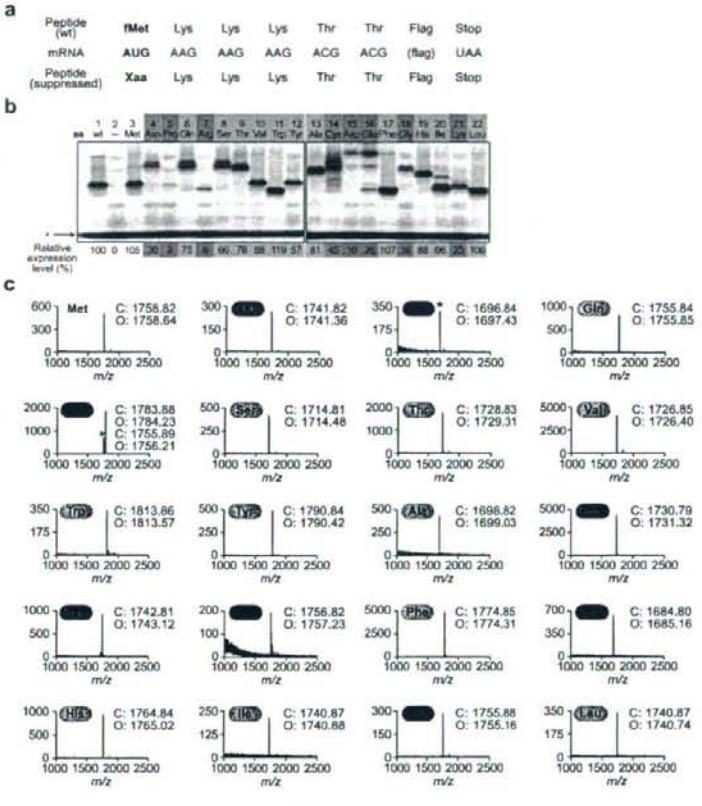

Figure 2. Tolerance of various amino acids in initiation. a) The mRNA sequence that expresses peptides initiated by various amino acids. Flag in parentheses indicates the RNA sequence encoding a Flag peptide (DYKDDDDK). b) Tricine-SDS PAGE analysis of the translation products. Lane 1, expression of wild type; lane 2, in the absence of Met; lane 3, initiated by Met-$tRNA^{fMet}_{CAU}$; lanes 4–22, initiated by Xaa-$tRNA^{fMet}_{CAU}$. Each expression level relative to wild type is determined by a mean score of triplicates or more. The band indicated by asterisk corresponds to the remaining [^{14}C]-Asp that was not incorporated into the peptide. The amino acids giving >50%, 30–50% and <30% of the wild-type expression level are highlighted in orange, pink, and cyan, respectively. Observed minor bands were likely attributed to peptides originated from the aa-$tRNA^{fMet}_{CAU}$-independent background expression, *i.e.*, random starts of translation of the RNA template. c) MALDI-TOF MS spectra of the translated peptides. The noncanonical amino acids are highlighted in the same color code as used in panel b. The red asterisk indicates a peak corresponding to the nonformylated peptide, while the unlabeled peak corresponds to the formylated peptides. The calculated mass (C) and observed mass (O) are shown in each spectrum.

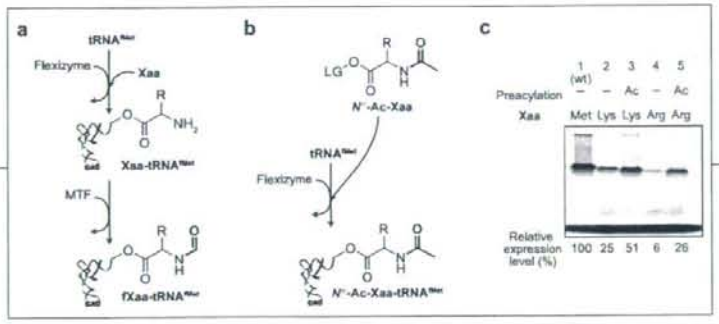

Figure 3. Increase in expression level by N^{α}-acylation. a) Formylation of Xaa-tRNA$^{fMet}_{CAU}$ in the *w*PURE system. When Xaa-tRNA$^{fMet}_{CAU}$ is used in the initiation, it is generally formylated. b) Aminoacylation of tRNA$^{fMet}_{CAU}$ with preacetylated amino acids by the flexizyme system. This method ensures N^{α}-acylation on the α-amino group. c) Tricine–SDS PAGE analysis of the translated peptide. Lane 1, expression of wild type; lane 2, initiated with Lys-tRNA$^{fMet}_{CAU}$; lane 3, initiated with N^{α}-Ac-Lys-tRNA$^{fMet}_{CAU}$; lane 4, initiated with Arg-tRNA$^{fMet}_{CAU}$; lane 5, initiated with N^{α}-Ac-Arg-tRNA$^{fMet}_{CAU}$. Each expression level relative to wild type is determined by a mean score of triplicates or more.

such a correlation based on experiments using an alternative codon for initiation. We therefore considered the possibility that preacylation on the α-amino group might increase the expression level of these peptides (Figure 3, panel b).

In order to verify this hypothesis, we chose two amino acids, Lys and Arg, both of which showed poor expression levels (25% and 6%, respectively) and prepared N^{α}-Ac-Lys-tRNA$^{fMet}_{CAU}$ and N^{α}-Ac-Arg-tRNA$^{fMet}_{CAU}$ by using the flexizyme system. It was investigated whether the expression level initiated with these preacylated amino acids increased compared with the corresponding α-amino-free amino acids. In both cases, peptide expression levels increased compared to those initiated with the corresponding α-amino-free amino acids (51% *vs* 25% for Lys and 26% *vs* 6% for Arg, Figure 3, panel c) and initiation proceeded exclusively with the designated N^{α}-Ac-amino acids (see Figure S1 for the MALDI-TOF molecular weight confirmation). It should be noted that even though the α-amino group on Arg was preacylated, the expression level of the peptide initiated with N^{α}-Ac-Arg did not exceed 50% efficiency compared with that of Met-initiated peptide. This suggests that other components in the *w*PURE system, such as IFs and ribosome, also play some roles in determining the initiation efficiency. Nonetheless, the preacylation on the α-amino group enhances the expression level for "modest" initiators.

Tolerance of N^{α}-Acyl Groups in Initiation. Biologically active peptides often have unique N-terminal-acyl groups. For instance, the N-terminus of some peptides synthesized by nonribosomal peptide synthetases are modified with fatty acids (*27*), while some peptide hormones have an acetyl or pyroglutamate group at their N-terminus (*28, 29*). Thus, it is of interest to see if the reprogrammed initiation in the *w*PURE system is able to tolerate amino acids with a variety of N^{α}-acyl groups. We chose Phe as an N^{α}-acyl carrier and expressed peptides initiated with Phe having alkyl or fatty acids (Ac, Hex, Mhe, Oct), pyroglutamate (pyE), and functionalities accessible to post-translational modification (Cba, Pen, Pyl, N_3Ac, oxP, ClAc; Figure 4, panel a).

Translation was initiated with N^{α}-acyl-Phe-tRNAs prepared by the flexizyme system, and the *N*-acyl-peptides were expressed in the *w*PURE system (Figure 4, panel b). Remarkably, the expression levels were generally higher than those of fMet- and fPhe-initiated control peptides; particularly the use of Phe modified with hydrophobic fatty acids gave significantly higher expression levels. MALDI-TOF analysis of these peptides agreed with the expected products without contamination of other misinitiated peptides (Figure 4, panel c). Thus, the results clearly indicated that a wide variety of N^{α}-acyl groups are tolerated in the initiation event. The N^{α}-acyl groups incorporated in this study involved various chemical functionalities such as fatty acid chains, pyroglutamate, and those that are applicable to post-translational modification. Hence, this approach would allow us to directly translate naturally occurring bioactive peptides containing unique N-terminal groups.

Ribosomal Synthesis of Cyclic Peptides *via* a Thioether Linkage. G7–18NATE is a potential peptidic anticancer agent, which has been shown to inhibit the interaction between the SH2 domain of Grb7 and receptor tyrosine kinases (*30–32*). The peptide consists of 11 natural amino acids and a non-natural cyclic structure with a nonreducible thioether bond linking the N-terminal acetyl group to the C-terminal Cys residue (see Figure S2a). Due to the fact that G7–18NATE contains a noncanonical bond, ordinary translation has been inapplicable to its synthesis; therefore this peptide could only be produced through chemical synthesis. However, we envisaged that our platform technology would enable us to perform the ribosomal synthesis of G7–18NATE.

We expected that reprogramming initiation with N^{α}-chloroacetyl (ClAc)-Trp would allow us to synthesize the linear N^{α}-ClAc-(G7–18NATE) precursor peptide, and subsequently the sulfhydryl group on the terminal Cys would spontaneously attack the α-carbon of the N^{α}-ClAc group, closing the ring (Figure 5, panel a) (*33–35*). We

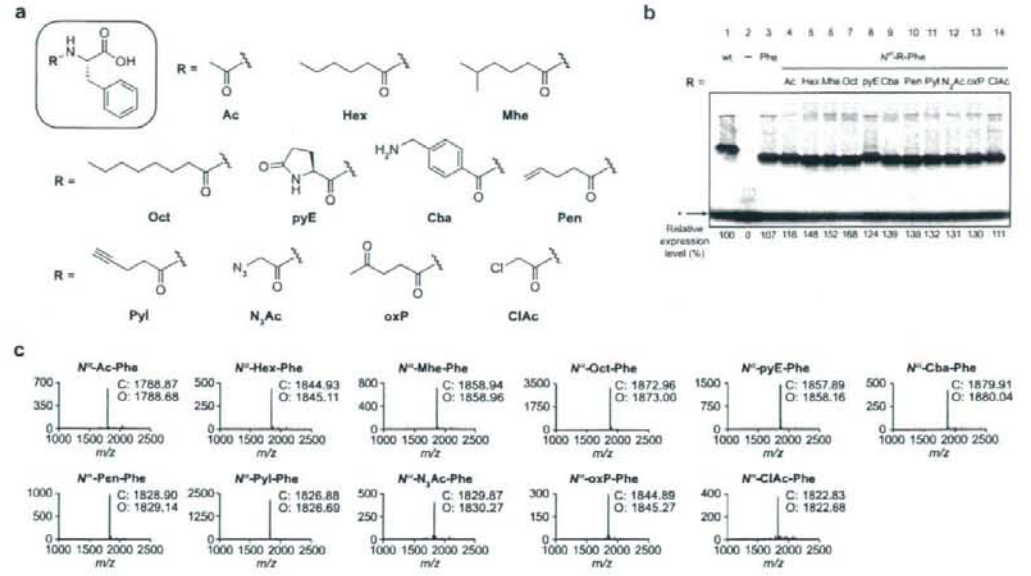

Figure 4. Tolerance of N^{ε}-acyl groups in initiation. a) N^{ε}-acyl phenylalanine derivatives used in this study. b) Tricine–SDS PAGE analysis of the translation products initiated with various N^{ε}-acyl phenylalanine derivatives. Lane 1, expression of wild type; lane 2, in the absence of Met; lane 3, initiated with Phe-tRNA$^{fMet}_{CAU}$; lanes 4–14, initiated with various N^{ε}-acyl-Phe-tRNA$^{fMet}_{CAU}$ molecules. Each expression level relative to wild type is determined by a mean score of duplicates. The band indicated by an asterisk corresponds to the remaining [^{14}C]-Asp that was not incorporated into the peptide. c) MALDI-TOF MS spectra of the translated peptides. The calculated mass (C) and observed mass (O) are shown in each spectrum.

thus designed a mRNA sequence to express the linear peptide initiated with N^{ε}-ClAc-Trp in the *w*PURE system (Figure 5, panel b). After expression, the resulting peptide was purified by molecular weight cut and desalting filters. MALDI-TOF analysis of the peptide gave peaks of the cyclic G7–18NATE with the expected molecular masses of $[M + H]^+$ and $[M + Na]^+$ (Figure 5, panel c), and MALDI-TOF/TOF analysis revealed peaks corresponding to the peptide fragments bearing the intact thioether linkage (Figure 5, panel d). This was a sharp contrast to the observation that no cyclization occurred in a control peptide in which the C-terminal Cys was substituted with Ser; *i.e.*, the N^{ε}-ClAc group did not react potential other sulfhydryl reagents such as mercaptoethanol (Figure S3). Remarkably, such a simple purification protocol yielded the desired cyclic peptide as the main product, while no peak corresponding to the unreacted linear N^{ε}-ClAc-(G7–18NATE) peptide was observed, suggesting that cyclization of the linear peptide occurred rapidly. Furthermore, although MALDI-TOF analysis does not permit the quantitative discussion, it should be noted that the undesired minor peaks, which could be caused by side reactions, *e.g.*, intermolecular reactions with other thiol molecules, such as DTT, mercaptoethanol, or free Cys, included in the *w*PURE system, were not observed in the mass spectrum. This indicated that the cyclization did not substantially suffer from such side reactions. In fact, because the intramolecular cyclization took place without undesirable side reactions, we were able to isolate the desirable full-length peptide through a simple purification procedure using a molecular weight cut filter.

Moreover, the expression level of G7–18NATE was quantified by the incorporated radioisotope counts of [^{14}C]-Asp calibrated against known [^{14}C]-Asp concentrations (see Figure S4). G7-18NATE was expressed at a

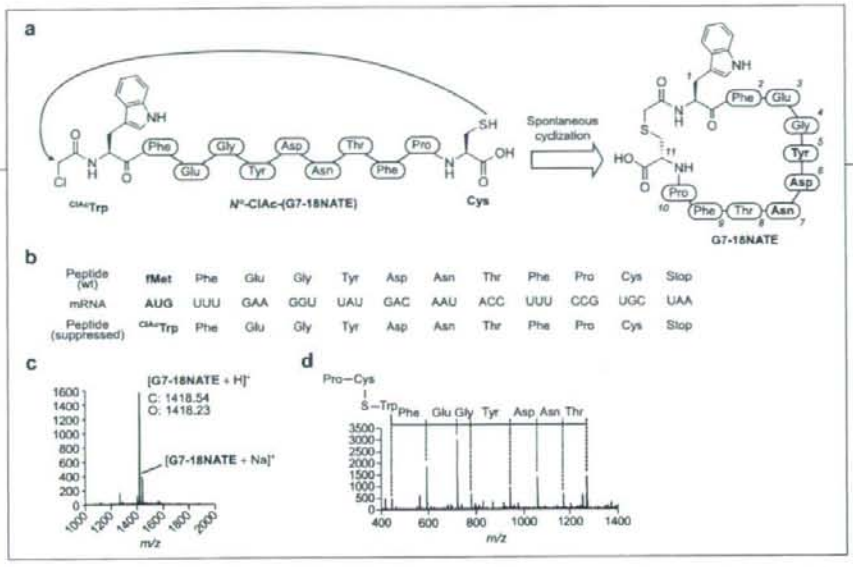

Figure 5. Ribosomal synthesis of G7−18NATE. a) The spontaneous cyclization affording G7−18NATE. The precursor linear peptide, N^2-ClAc-G7−18NATE, translated in the *w*PURE system spontaneously cyclizes into G7-18NATE by intramolecuar attack of Cys sulfhydryl group to the α-carbon of the N^2-ClAc group. b) The sequence of mRNA used in the ribosomal synthesis of G7−18NATE. For the synthesis of G7−18NATE, the start codon is suppressed with N^2-ClAc-Trp. c) MALDI-TOF mass spectrum of the expressed G7−18NATE. The calculated mass (C) and observed mass (O) are shown in the figure. The peaks at 1263.97 and 1401.23 *m/z* were occasionally observed when the molecular weight cut purification protocol was utilized, regardless of template sequence. Therefore, it is likely that these peaks originated from components in the *w*PURE system. d) MALDI-TOF/TOF analysis of G7−18NATE expressed in the *w*PURE system. The representative peaks that correspond to the peptide fragments containing the thioether linkage are labeled in the spectrum.

concentration of approximately 3.7 μM (5.3 μg/mL), which is comparable to the control expression initiated with Met generated by the normal PURE system (~5 μM), indicating that the reprogrammed initiation was achieved without significant reduction in expression level.

To further examine the flexibility of this strategy for the synthesis of cyclic peptides, we designed three peptide sequences based on the structure of G7−18NATE with arbitrarily chosen ring sizes (Figure 6, panel a). Three amino acids (Tyr, Asp, and Asn), which are the most critical residues for the binding activity to Grb7, were kept in the structural designs of these cyclic peptides (*30*), and the ring size was varied from 11 (as in the original G7−18NATE) to 4, 6, or 14 residues. These cyclic peptides were designed to include a modified Flag peptide at the C-terminus in order to ensure MALDI-TOF ionization. MALDI-TOF analysis of the translation products showed that each of the expressed peptides spontaneously cyclized upon translation of the corresponding linear peptide (Figure 6, panel b). This result suggested that the *in situ* cyclization process is independent of peptide sequence and ring size. A limitation of this technology is likely that the peptide can have only a single Cys residue in order to avoid undesirable competing cyclization reactions, but yet it is widely applicable to a variety of sequences of cyclic peptides.

It should be noted that G7−18NATE was an engineered synthetic peptide designed based on the parental peptide (G7−18NA) (*30*). G7−18NA was originally selected by phage display and thus composed of proteinogenic amino acids linked with a Cys−Cys disulfide bond (see Figure S2b). Despite the fact that the engineering of G7−18NA to G7−18NATE reduced the binding activity to Grb7, its *in vivo* stability resulted in successful suppression of pancreatic cancer metastasis in mice (*30*–*32*). Ribosomal synthesis of G7−18NATE would enable us to construct a mRNA-programmed G7−18NATE-like peptide library for reoptimizing the binding to Grb7. Particularly, the coupling of this technology with an *in vitro* display system (*36*–*38*) would allow us to rapidly screen the above library without sacrificing the peptide's *in vivo* compatibility.

Construction of a Combinatorial Cyclic Peptide Library. We have demonstrated the generality of the *in situ* cyclization of peptides with various sequences and ring sizes. This indicates that a variety of *in vivo* compatible cyclic peptide libraries can be readily prepared by simply designing mRNA sequence libraries. As such a demonstration, we have constructed a peptide library consisting of 160 distinct cyclic peptides. We prepared 160 DNA templates, coding 6-mer peptides bearing Cys at the C-terminus, each of which has two varying codons assigning Xaa1 and Xaa2 (Figure 7, panels a and b, and Figure S4); then, peptides were expressed in parallel from the respective DNA templates in the presence of N^2-ClAc-Tyr-$tRNA^{fMet}_{CAU}$ using the *w*PURE sys-

tem. To confirm the quality of products, 12 samples were randomly chosen from the library and were passed through a desalting filter. Remarkably, even without vigorous purifications, we were able to detect the desired cyclic peptide with the expected molecular weight as a sole product in all samples (Figure 7, panel c). This demonstration proves the practicality of this technology for the synthesis of cyclic peptide libraries closed by the nonreducible thioether bond. We are currently pursuing the screening of extended cyclic peptide libraries prepared by this platform technology against chosen therapeutic targets.

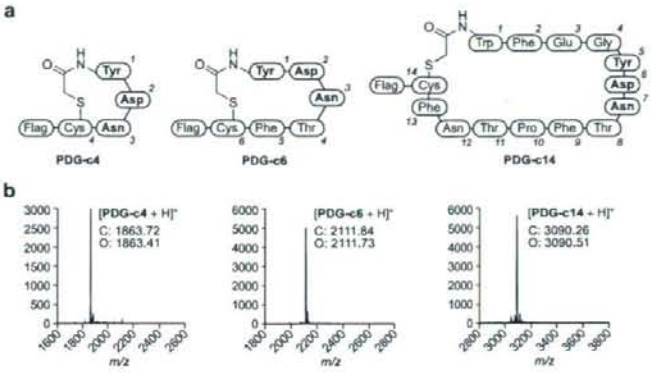

Figure 6. Custom synthesis of the cyclic peptides with various ring sizes. a) Structure of the expressed cyclic peptides. Three amino acids known to be critical residues in G7−18NATE are shown in bold, and "Flag-tag" indicates a modified Flag peptide sequence (LTTDYKDDDDK). b) MALDI-TOF mass spectrum of the expressed cyclic peptides. The calculated mass (C) and observed mass (O) are shown in the spectra.

CONCLUSION

In conclusion, we have applied the concept of genetic code reprogramming to the engineering of the initiation event in translation by means of the *w*PURE and flexizyme systems. The translation apparatus surprisingly tolerates all proteinogenic amino acids in the initiation event, particularly those having hydrophobic side chains with high efficiencies. It also accepts a wide array of N^{α}-acyl groups containing various functionalities. This technology has been applied to the ribosomal synthesis of cyclic peptides linked *via* a physiologically stable thioether bond, where the cyclization occurs spontaneously upon translation of the precursor linear peptide. Indeed, we have demonstrated the construction of a high-quality library consisting of 160 distinct cyclic peptides by simply designing a mRNA library. Because this new platform technology is widely applicable to the synthesis of various cyclic peptide libraries containing not only natural but also nonproteinogenic amino acids using reprogramming of the elongation event, it is a powerful tool to accelerate the discovery of peptidic drug candidates against various therapeutic targets.

METHODS

General Protocol of Translation. Xaa-tRNA$^{fMet}_{CAU}$ was prepared by the following procedure. tRNA$^{fMet}_{CAU}$ (40 μM) in 0.2 M Hepes-K (Hepes, 2-[4-(2-hydroxyethyl)-1-piperadinyl]ethansulfonic acid) buffer pH 7.5, 0.2 M KCl (7.5 μL) was heated at 95 °C for 3 min and cooled to 25 °C for 5 min. $MgCl_2$ (3 M, 3 μL) and flexizyme (dFx or eFx, see ref (*20*)) (200 μM, 1.5 μL) were added, and the mixture was incubated at 25 °C for 5 min. The reaction was initiated by addition of 3μL of 25 mM substrate (N^{α}-acyl-amino acid 3,5-dinitrobenzyl ester or cyanomethyl ester) in dimethyl sulfoxide and incubated on ice for the optimized times, generally 2−6 h (*20*). After acylation, the reaction was stopped by addition of 45 μL of 0.6 M sodium acetate at pH 5, and the RNA was recovered by ethanol precipitation. The pellet was rinsed twice with 70% ethanol with 0.1 M sodium acetate, pH 5.0, and once with 70% ethanol. The Xaa-tRNA$^{fMet}_{CAU}$ was dissolved in 0.5 μL of 1 mM sodium acetate just before adding to translation mixture.

The *w*PURE system containing all necessary components for translation except for all 20 standard amino acids was used in this study. Translation was carried out using *w*PURE system with 0.04 μM mDNA1 containing 200 μM each Thr, Tyr, and Lys 50 μM [^{14}C]-Asp, and 120 μM of various Xaa-tRNA$^{fMet}_{CAU}$ molecules. The wild-type expression was carried out with *w*PURE system with 0.04 μM mDNA1 and 3 mM ethylenediamine tetraacetic acid containing 200 μM each Thr, Tyr, and Lys and 50 μM [^{14}C]-Asp. The translation mixture (2.5 μL) was incubated at 37 °C for 1 h and analyzed by Tricine−SDS-PAGE and autoradiography (FLA-5100, Fuji, Japan).

Mass Spectra Measurements of Peptides. For mass spectra analysis, translation reaction (5 μL) was performed in the presence of Asp, instead of [^{14}C]-Asp. The translation product from mDNA1 was immobilized with FLAG-M2 agarose (Sigma). After the resin was washed with 30 μL of W buffer (50 mM Tris-HCl (Tris, tris(hydroxymethyl)aminomethane) pH 8.0, 150 mM NaCl), the immobilized peptides were eluted with 10 μL of 0.2% trifluoroacetic acid (TFA). The purified peptide was desalted with ZipTip$_{\mu\text{-C18}}$ (Millipore), and eluted with 1 μL of a 50% acetonitrile, 0.1% TFA solution saturated with the matrix (*R*)-cyano-4-hydroxycinnamic acid. MALDI MS measurement was performed

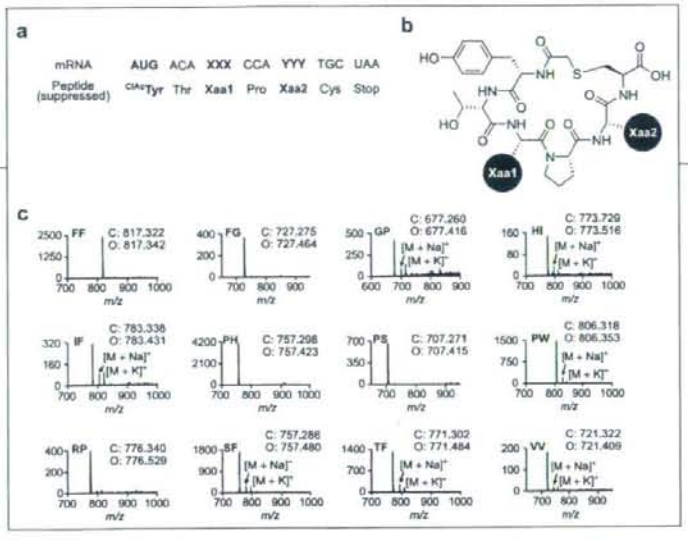

Figure 7. Construction of a cyclic peptide library. a) The sequence of mRNA used in the library synthesis. The codons indicated in red (Xaa1) and blue (Xaa2) were changed in each expression. The start codon is suppressed with *N*$^{\alpha}$-ClAc-Tyr. b) The structure of cyclic peptide contained in the peptide library. c) MALDI-TOF analysis of the components of the peptide library. The amino acids corresponding to Xaa1 and Xaa2 were shown in red and blue in each spectrum, respectively. A one-letter amino acid abbreviation was used. The peaks labeled by $[M + Na]^+$ and $[M + K]^+$ correspond to sodium adduct and potassium adduct of the desirable product, respectively. The calculated mass (C) and observed mass (O) are shown in each spectrum.

using autoflex II TOF/TOF (Bruker Daltonics) under the linear/positive mode and externally calibrated with peptide calibration standard II (Bruker Daltonics).

Ribosomal Synthesis of G7–18NATE. Translation was carried out using *w*PURE system with 0.04 μM mDNA2 containing 200 μM each Phe, Glu, Gly, Tyr, Asn, Thr, Pro, Asp, and Cys and 120 μM *N*$^{\alpha}$-ClAc-Trp-tRNA$^{fMet}_{CAU}$. The translation mixture (5 μL) was incubated at 37 °C for 1 h. The product was diluted three times with water and purified through Microcon YM-10 (Millipore) (10,000 MW cut filter). The purified peptides were desalted with ZipTip$_{\mu\text{-C18}}$ (Millipore) and eluted with 1 μL of a 50% acetonitrile, 0.1% TFA solution saturated with the matrix (*R*)-cyano-4-hydroxycinnamic acid. MALDI-MS measurements were performed using autoflex II TOF/TOF (Bruker Daltonics) under the linear/positive mode and externally calibrated using peptide calibration standard II (Bruker Daltonics). MALDI-TOF/TOF analysis was also carried out using autoflex II TOF/TOF under the lift mode.

Construction of Combinatorial Cyclic Peptide Library. Translation reactions were carried out in parallel using 96-well plates. 0.04 μM DNA templates were mixed with *w*PURE system containing 22 μM *N*$^{\alpha}$-ClAc-Trp-tRNA$^{fMet}_{CAU}$. The expressions of samples #1–144 were performed with aa mix A (Ile, Ala, Gly, Leu, Val, Pro, Asn, Ser, Thr, Tyr, Phe, and Trp; 500 μM each), and the expressions of sample #145–160 were performed with aa mix B (Pro, Arg, Asp, Glu, Gln, His, Ile, Ala, Thr, Tyr, Asn; 500 μM each). The translation mixture (40 μL) was incubated at 37 °C for 1 h. For characterization of the product, randomly selected samples (#11, 30, 53, 68, 72, 95, 107, 123, 131, 149, 150, and 156) were analyzed by MALDI-TOF mass spectrometry. The translation product (3 μL) was acidified by adding 5 μL of 1% TFA and desalted with ZipTip$_{\mu\text{-C18}}$ (Millipore). The sample was eluted with 1 μL of a 0.1% TFA solution containing 50% acetonitrile and 2,5-dihydroxybenzoic acid (10 mg mL^{-1}) onto MTP 384 target plate ground steel T F (Bruker Daltonics). MALDI-MS measurements were performed using autoflex II TOF/TOF under the linear/positive mode and externally calibrated using peptide calibration standard II (Bruker Daltonics).

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

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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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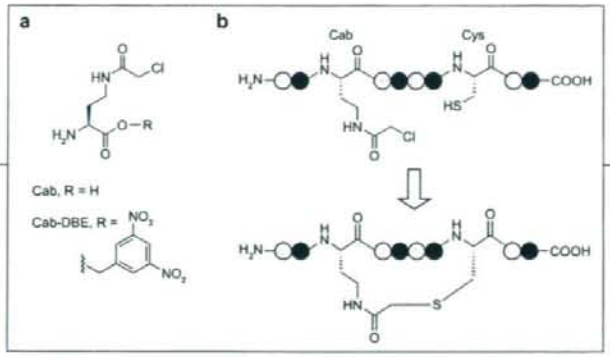

Figure 1. Incorporation of Cab into a peptide chain followed by inter-side-chain cyclization. (a) Chemical structure of N^{γ}-(2-chloroacetyl)-α,γ-diamino-butylic acid (Cab) and 3,5-dinitrobenzyl ester (DBE) form of Cab as a substrate for dFx (dinitro-Flexizyme). (b) Schematic representation of peptide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

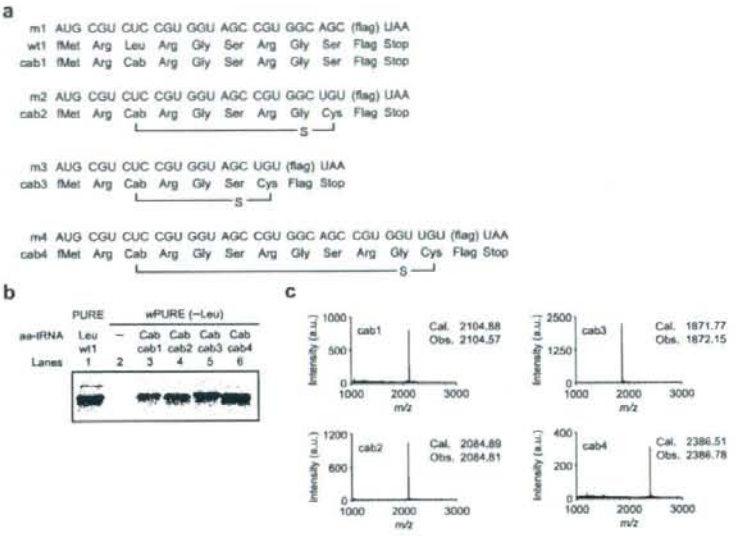

Figure 2. Incorporation of Cab into a peptide chain and its spontaneous cyclization. (a) Sequence of mRNAs (m1–4) and corresponding peptides (wt and cab1–4). Flag in parentheses indicates the RNA sequence encoding the Flag peptide. (b) Tricine SDS-PAGE analysis of the translated products labeled with [^{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

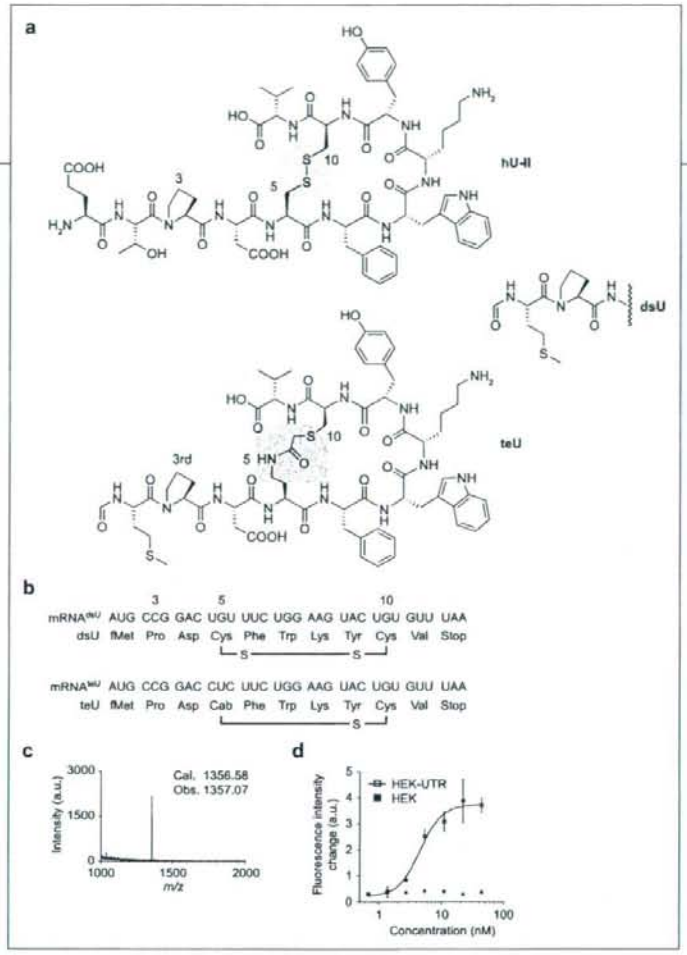

Figure 3. Expression of human urotensin II-like peptides. (a) Molecular structures of human urotensin II (hU-II), dsU, and teU. The inter-side-chain bond in each peptide is highlighted in a gray rectangle. (b) Sequences of mRNA templates encoding human urotensin II-like peptide, dsU (disulfide containing 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 of the peptide, purification, concentration estimation, and biological assay are reliable to evaluate hU-II-like peptides.

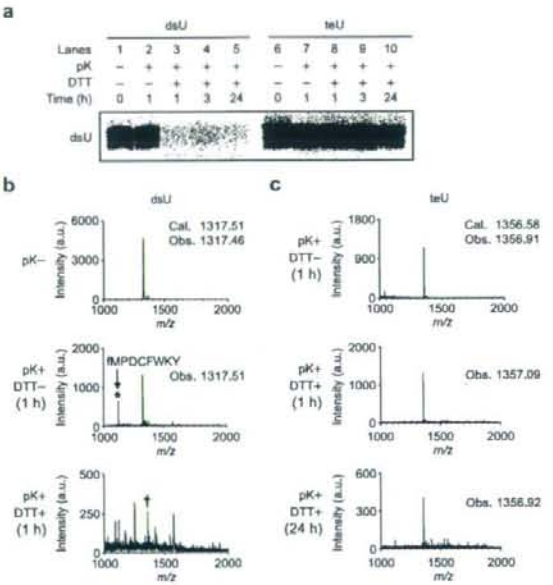

Figure 4. Analysis of peptide stability by proteinase K (pK). (a) Tricine SDS-PAGE analysis of dsU and teU incubated with pK. Peptides were labeled with [^{14}C]-Val and detected by autoradiography. dsU and teU were analyzed without pK (lanes 1 and 6) or with pK (lane 2–5 and 7–10), in the absence of 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.

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