The Cyclization and Polymerization of Bacterially Expressed Proteins Using Modified Self-splicing Inteins

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Mini-inteins derived from Synechocystis sp. (Ssp DnaB intein) and Mycobacterium xenopi (Mxe GyrA intein) that have been modified to cleave peptide bonds at their C and N termini, respectively, were cloned in-frame to the N and C termini of a target protein. Peptide bond cleavage of the modified inteins generated an N-terminal cysteine and a C-terminal thioester on the same protein. These complementary reactive groups underwent intra- or intermolecular condensation to generate circular or polymeric protein species with a new peptide bond at the site of ligation. Three cyclic peptides, BBP, an organ specific localization peptide; RGD, an inhibitor of platelet aggregation; and CDR-H3/C2, which inhibits an organ specific localization peptide; RGD, an inhibitor of platelet aggregation (21), and inhibition of HIV-1 replication (22). Furthermore, future studies on protein polymerization may allow the production and investigation of analogs of fibrous proteins, such as silk.

EXPERIMENTAL PROCEDURES

TWIN Vector Construction—All vectors are derived from pTYB1 (New England Biolabs) or pTXB1 (7). The TWIN vectors utilize the Ssp DnaB mini-intein mutated for C-terminal cleavage (8), Ssp mini-intein (Cys9→Ala) (a 154-aa intein derived from the Synechocystis sp. DnaB gene with a Cys9→Ala mutation), and the Mxe GyrA intein (Asn198→Ala) (an intein in the Mycobacterium xenopi GyrA gene with an Asn198→Ala mutation), modified for N-terminal cleavage (7). pSBX1 was composed of the Ssp mini-intein (Cys9→Ala) followed by a multiple cloning site (MCS) (5-G'AGA GGC ATG CAA TTC TCG TCG ACG GCG GCC GCC TCG AGG GCT CTT CCC-3'), the Mxe GyrA intein (Asn198→Ala) and the chitin binding domain (CBD) from Bacillus circulans (23). Plasmid pSTX1 contains the gene for Escherichia coli thioredoxin with coding sequence for 3 amino acids added to the N and C termini of the protein: Cys-Gly-Gly and Met-Arg-Met, respectively, inserted between the two inteins in pSBX1.

pBSBX1 and pBSBX2 are TWIN vectors that place a CBD at the N and C termini of the precursor protein. pBSBX1 encodes for a fusion protein of the CBD-Ssp mini-intein (Cys9→Ala)-MCS-Mxe GyrA intein (Asn198→Ala)-CBD, where the MCS is 5-G'AGA GGC ATG CAA TTC TCG TCG ACG GCG GCC GCC TCG AGG GCT CTT CCC-3', pBSBX2 is identical to pBSBX1 except that the MCS region is 5-TGG CCG GCC ATG GGC GCG CGC AAT GGA AGA GCT CTA GCG ACA ACA ACA ACA ACA ACA ACA ACA ACA ACAA ACA AGG TCG TGG AGG GAA GCG GTA CGC TCG AGG GC-3'. pBSBX2 encodes for a factor Xa (FXa) cleavage site 5 amino acids upstream from the N terminus of the Mxe GyrA intein. Insertion of the E. coli malE sequence, encoding maltose binding protein (MBP) (24), between the NcoI to SacI sites in pBSBX2 yielded pBSM8X1. pBSTBX1 has the thioredoxin gene cloned in place of MBP in pBSM8X1. MBP purified from pBSM8X1 and thioredoxin purified from pBSTBX1 have Cys-Arg-Ala and Ser-Ser-Asn10-Leu-Gly-Ile-Glu-Gly-Ary-Gly-Thr-Leu-Glu-Gly added to their N and C termini, respectively.

Cloning of the thioredoxin gene into the AgeI to PstI sites in pBSL-C155 (8) created pBST1. pSTX6 was then generated by cloning the NdeI to RsrII fragment from pBST1 into the same sites in pSTX1. These constructs have DNA encoding Cys-Arg-Ala-Met-Gly-Gly-Ary-Thr-Gly and Met-Arg-Met added to the N and C termini of thioredoxin.
Yields of peptides from pBBP1, pRGD1, and pCDR1 were 20–50 mg/liter of cell culture when MESNA was used to induce cleavage of intein 2. However, the yields were 0.5–1 mg/liter of cell culture when NH₂OH replaced MESNA as the cleavage reagent. Protein Purification from TWIN Vectors—ER2566 cells containing the appropriate TWIN plasmid were grown, induced, and pelleted as described for the Mxe GyrA intein (Asn₁⁰⁹⁶ → Ala) (7). The pellets were resuspended in Buffer A (20 mM Tris-HCl, pH 8.5, containing 500 mM NaCl). Following sonication of the cell pellet, debris was removed by centrifugation at 23,000 × g for 30 min. This clarified supernatant was applied to a chitin resin (bed volume, 15 ml) equilibrated in Buffer A. Cleavage of Intein 1 (see Fig. 1), Ssp mini-intein (Cys¹ → Ala), was initiated by equilibrating the chitin column in Buffer B (20 mM Tris, pH 7.0, containing 500 mM NaCl) and proceeded for 20 h at room temperature, after which the resin was washed with 10 column volumes of Buffer B (500 mM NaCl). Following cleavage of Intein 2, Mxe GyrA intein (Asn₁⁰⁹⁶ → Ala), was performed by equilibrating the chitin resin in Buffer C (50 mM Tris, pH 8.5, containing 100 mM 2-mercaptoethanesulfonic acid (MESNA) and 250 mM NaCl) and incubating overnight at 4 °C. The released target protein was eluted from the chitin resin using Buffer C. Purification with pSTX1 omitted the Intein 1 cleavage step, as this occurred in vivo, and Buffer D (50 mM Tris, pH 7.4, containing 30 mM NH₂OH and 500 mM NaCl) replaced Buffer C.

Total yields of protein from pSTX1, pSTX6, pSTBX1, and pBSMXB1 were 5–15 mg/liter of cell culture when MESNA was used to induce cleavage of intein 2. However, the yields were 0.5–1 mg/liter of cell culture when NH₂OH replaced MESNA as the cleavage reagent. Yields of peptides from pBBP1, pRGD1, and pCDR1 were 20–50 μg/liter of cell culture. Thioredoxin concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as the standard. Peptide concentrations were determined by comparing the absorbance at 214 nm of the peptide solution to the absorbance at 280 nm and using a molar absorptivity of 14,100. MBP concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as the standard. Peptide concentrations were determined by comparing the absorbance at 214 nm of the HPLC eluted peptide peaks with a 13-amino acid peptide, NH₂-ThrPhePheNH₂-COOH, of known concentration. The peptide solution was bound to a Vydac 218TP51 column and eluted with a 1 to 75% acetonitrile gradient; all HPLC solutions contained 0.1% trifluoroacetic acid.

Protein Circularization, Polymerization, and Ligation—On-column protein cyclization reactions occurred when MESNA was used to induce cleavage of intein 2 as described under “Protein Purification from TWIN Vectors.” Following elution from the chitin resin, the cyclic proteins were investigated with either 10–20% Tricine gels (Novex) or MALDI-TOF mass spectrometry (a PerSeptive Biosystems Voyager-DE Biospectrometry workstation). Incomplete cyclization of thioredoxin allowed multimerization of noncircular molecules. Multimerization was accelerated by concentrating the freshly purified protein in a Centricprep 10 followed by a Centricron 10 concentration apparatus (Amicon) to a final concentration of total protein of 54 mg/ml. Ligation of thioredoxin to maltose binding protein utilized thioredoxin derived from pSTX1 and NH₂OH as the cleaving reagent. The thioredoxin was extensively dialyzed against Buffer E (10 mM Tris, pH 7.4, containing 100 mM NaCl) to remove unreacted NH₂OH. The dialyzed thioredoxin was mixed with freshly isolated thiostere-tagged MBP, purified using plasmid pHMB10G as described previously (14), and allowed to react overnight at 4 °C. The final concentration of the ligating species was 4.5–13.7 mg/ml thioredoxin and 1–3 mg/ml thioster-tagged MBP. Polymerization and ligation reactions were visualized by SDS-PAGE on 12% Tris-glycine or 10–20% Tricine gels (Novex) stained with Coomassie Brilliant Blue. Following polymerization, the reaction was subjected to SDS-PAGE using 10–20% Tricine gels. The bands were blotted onto nitrocellulose, and the three fastest migrating species were subjected to amino acid sequencing using a Procise 494 protein sequencer (PE Applied Biosystems, Foster City, CA).

Proteolysis and Sequencing of Circular Proteins—Plasmids pSTXB1 and pBSMXB1 encode thioredoxin and MBP, respectively, with a FXA site 5 amino acids from the predicted C terminus. Expression of these genes generated both linear and circular forms of the protein which were treated with FXAs (1:20; FXA:protein mass ratio) overnight respectively.

FIG. 1. TWIN System. The TWIN system sandwiches a target protein (T) between two inteins and can produce an N-terminal cysteine and a C-terminal thioester on the same bacterially expressed protein. Intein 1, Ssp mini-intein (Cys¹ → Ala), undergoes cleavage at its C terminus by cyclization of Asn¹⁰⁹⁶, whereas Intein 2, Mxe GyrA intein (Asn¹⁰⁹⁶ → Ala), undergoes thiol-induced cleavage of a thioester bond formed by a N-S acyl shift at Cys¹. Purification of the precursor protein is simplified by using a CBD, which binds to chitin resin. Cleavage at the C terminus of Intein 1, either in vitro or in vivo, releases an N-terminal cysteine on the target protein. A thiol reaction, such as MESNA, induces cyclization of Intein 2 and releases the target protein with an activated C-terminal thioester. Two reaction pathways can occur, either an intermolecular reaction that leads to polymerization or an intramolecular reaction to generate a circular protein. In both possibilities a peptide bond is formed during the ligation reaction at 4 °C. The proteolyzed proteins were run on 10–20% Tricine gels and blotted onto nitrocellulose, and the individual bands were subjected to amino acid sequencing.

RESULTS

Purification of Proteins Using the TWIN System—The TWIN system places an intein at both the N and the C termini of a target protein (Fig. 1). The intein at the N terminus of the target protein, Intein 1, was the Ssp mini-intein (Cys¹ → Ala), which contains a mutation that blocks protein splicing but allows cleavage at its C terminus (8). Intein 2, at the C terminus of the target protein, was the Mxe GyrA intein (Asn¹⁰⁹⁶ → Ala) that undergoes thiol-inducible cleavage at its N terminus (7). A chitin binding domain present on one or both of the inteins allowed the immobilization of the desired precursor protein in chitin resin, whereas endogenous E. coli proteins could be washed away (Fig. 2A, lane 2). Intein 1 was found to undergo either in vitro (Fig. 2A) or in vivo (Fig. 3, lane 2) cleavage with Cys-Arg or Cys-Gly at the N terminus of the target protein, respectively. In either case, an N-terminal cysteine was generated on the target protein. Cleavage of Intein 2 was induced with either MESNA or NH₂OH. Following cleav-
using NH$_2$OH, which blocks the cyclization reaction.

After NH$_2$OH-induced cleavage of Trx-thioredoxin, with an N-terminal cysteine, eluted from the chitin resin clarified cell extract after application to a chitin resin. Trx-Mxe precursor protein binds to the chitin resin. Incubated with NH$_2$OH, which blocks the cyclization reaction. Lane 4, thioredoxin produced using NH$_2$OH, which blocks the cyclization reaction. Lane 5, thioredoxin eluted after MESNA treatment was concentrated to 54 mg/ml and incubated overnight at 4 °C. The thioredoxin monomer is labeled l-Trx with multimers labeled sequentially, i.e. a dimer is Trx$_2$, and a trimer is Trx$_3$. B, lane 1, thioredoxin, from pBSTXB1, with a FXa site 5 amino acids from its C terminus. The putative circular thioredoxin migrates faster than the linear form. Lane 2, identical to lane 1 with NH$_2$OH used to prevent the cyclization reaction. Lane 3, thioredoxin from lane 1 incubated with factor Xa (1:20 FXa:thioredoxin), which proteolyses the c-Trx and converts it into a linear form. C, lane 1, MBP, from pBSMXB1, expressed with a FXa site 5 amino acids from its C terminus. The putative cyclic MBP (c-MBP) migrates slower than the linear form (l-MBP). Lane 2, linear MBP eluted following cleavage of Intein 2 with NH$_2$OH, which blocks the cyclization reaction. Lane 3, FXa cleavage (1:20 FXa:MBP) of the fraction described in lane 1. Following proteolysis the cyclic MBP co-migrates with l-MBP. All reactions were performed as described under "Experimental Procedures."

Cyclization of thioredoxin (135 aa) and MBP (395 aa) was investigated by incubating these proteins with FXa followed by amino acid sequencing. Proteins expressed with both pBSTXB1 and pBSMXB1 have a FXa site 5 amino acids from their C terminus. FXa treatment of the elution fractions resulted in the disappearance of the putative cyclic protein species when visualized on SDS-PAGE (Fig. 2, B and C). The cyclization reaction occurred to >80%, with 9 and 3 amino acids added to the N and C termini of thioredoxin, respectively (Fig. 2A, lane 3). When thioredoxin and MBP had 3 and 23 residues added to the N and C termini, respectively, cyclization was about 50% (Fig. 2, B and C).

Amino acid sequencing of the FXa cleaved samples yielded two sequences for thioredoxin, one expected for the linear, XRAMGDKIIGLTTD (predicted linear form is CRAMGDKIIGLTTD) and the other expected for the FXa linearized circular form, GTLEGCRAMGDKI, where GTLEG is the sequence expected at the C terminus of thioredoxin and CRAMGD is the expected N-terminal sequence. Two sequences were also detected for the FXa-treated cyclization reaction of MBP, XRAMGIEEGKL, which matched the expected N-terminal sequence for the linear MBP (CRAMGIEEGKL), and XTLEGCRAMGII, which agreed with the predicted sequence for the linearized cyclic MBP where GTLEG is the expected C-terminal sequence of MBP and CRAMGI is the predicted N-terminal sequence. In the amino acid sequencing data, an X is used to indicate that an amino acid could not be assigned for that sequencing cycle.

**Cyclization Peptides**—The cyclization of the small peptides BBP (9 aa), RGD (10 aa), and CDR-H3/C2 (14 aa) were confirmed by mass spectrometry (Table I and Fig. 4B). Predicted molecular masses for cyclic BBP, RGD, and CDR-H3/C2 were 977.2, 1120.3, and 2098.3 g/mol, respectively. These agreed well with the experimentally determined values of 977.1, 1119.9, and 2098.7 g/mol, respectively. A linear peptide generated by hydrolysis of the C-terminal thioester was not observed using the MALDI-TOF mass spectrometer. However, when using pBP1 an extra species was observed with an apparent molecular mass of 1145.3 g/mol. This molecular mass is greater than the expected mass of the MESNA-tagged peptide, 1119.3 g/mol, and may represent the thioester-tagged linear BBP peptide that has undergone either in vitro or in vivo modification. However, this was not verified and peptides from pRGD or pCDR had no detectable levels of a comparable species.

**Protein Polymerization**—Polymeric species of thioredoxin were generated by concentrating freshly isolated thioredoxin, purified from cells containing pSTX6, as described under "Experimental Procedures." Multiple bands were visible that corresponded to the expected molecular masses of multimers of

| Protein sample | Predicted linear molecular mass | Predicted circular molecular mass | Observed molecular mass |
|----------------|---------------------------------|----------------------------------|-------------------------|
| BBP            | 995.2                           | 977.2                            | 977.1                   |
| RGD            | 1138.3                          | 1120.3                           | 1119.9                  |
| CDR-H3/C2      | 2116.3                          | 2098.3                           | 2098.6                  |

**FIG. 2.** Circularization and polymerization reactions. A, lane 1, clarified cell extract showing the precursor protein, CBD-Ssp (Cys$_3^{1} \rightarrow$ Ala)-Trx-Mxe (Asn$_{180}^{1}$ \rightarrow Ala)-CBD (62 kDa), from pSTX6. Lane 2, clarified cell extract following passage over a chitin column. Note that the precursor protein binds to the chitin resin. Lane 3, elution of thioredoxin following pH and temperature-dependent cleavage of Intein 1 and MESNA-induced cleavage of Intein 2. Both linear (l-Trx) and cyclic (c-Trx) forms of thioredoxin are visible. Lane 4, thioredoxin produced using NH$_2$OH, which blocks the cyclization reaction. Lane 5, thioredoxin eluted after MESNA treatment was concentrated to 54 mg/ml and incubated overnight at 4 °C. The thioredoxin monomer is labeled l-Trx, with multimers labeled sequentially, i.e. a dimer is Trx$_2$, and a trimer is Trx$_3$. B, lane 1, thioredoxin, from pBSTXB1, with a FXa site 5 amino acids from its C terminus. The putative circular thioredoxin migrates faster than the linear form. Lane 2, identical to lane 1 with NH$_2$OH used to prevent the cyclization reaction. Lane 3, thioredoxin from lane 1 incubated with factor Xa (1:20 FXa:thioredoxin), which proteolyses the c-Trx and converts it into a linear form. C, lane 1, MBP, from pBSMXB1, expressed with a FXa site 5 amino acids from its C terminus. The putative cyclic MBP (c-MBP) migrates slower than the linear form (l-MBP). Lane 2, linear MBP eluted following cleavage of Intein 2 with NH$_2$OH, which blocks the cyclization reaction. Lane 3, FXa cleavage (1:20 FXa:MBP) of the fraction described in lane 1. Following proteolysis the cyclic MBP co-migrates with l-MBP. All reactions were performed as described under "Experimental Procedures."

**FIG. 3.** IPL reaction with an N-terminal cysteine generated in vivo. Lane 1, uninduced cell extract. Lane 2, clarified cell extract of the thioredoxin precursor protein, Ssp (Cys$_3^{1} \rightarrow$ Ala)-Trx-Mxe (Asn$_{180}^{1}$ \rightarrow Ala)-CBD (58 kDa), expressed from pSTX1. Intein 1 has cleaved in vivo generating Trx-Mxe-CBD (39 kDa) and Ssp (Cys$_3^{1} \rightarrow$ Ala) (Ssp, 18.6 kDa). Lane 3, clarified cell extract after application to a chitin resin. Trx-Mxe-CBD binds to the resin, whereas Ssp, lacking a CBD, flows through. Lane 4, thioredoxin, with an N-terminal cysteine, eluted from the chitin resin after NH$_2$OH-induced cleavage of Trx-Mxe-CBD. Lane 5, MBP, with a C-terminal thioester, purified separately from pMRB10G (14). Lane 6, ligation reaction of thioredoxin (13.5 mg/ml) and MBP (3 mg/ml) as described under "Experimental Procedures." A new band, corresponding to thioredoxin ligated to MBP (MBP-Trx), is visible.

**Table I**

| Protein sample | Predicted linear molecular mass | Predicted circular molecular mass | Observed molecular mass |
|----------------|---------------------------------|----------------------------------|-------------------------|
| BBP            | 995.2                           | 977.2                            | 977.1                   |
| RGD            | 1138.3                          | 1120.3                           | 1119.9                  |
| CDR-H3/C2      | 2116.3                          | 2098.3                           | 2098.6                  |
The facile isolation of cyclic proteins possessing a continuous peptide bond backbone is accomplished using one affinity chromatography step with the TWIN system (Fig. 1). The TWIN system sandwiches a target protein between two inteins, one engineered to cleave at its C terminus (Intein 1) and the other modified to undergo thiol-induced N-terminal cleavage (Intein 2). Cleavage of Intein 1 produces an N-terminal cysteine on a bacterially expressed protein, whereas thiol-induced cleavage of Intein 2 can produce a C-terminal thioester on the same protein. These reactive groups undergo spontaneous condensation as described previously (12, 13).

Three circular peptides were isolated with the TWIN system. BBP has been reported to target phage to specific organs in mice (20), RGD inhibits platelet aggregation (21), and CDR-H3/C2 has been shown to inhibit HIV-1 replication (22). Previously, these peptides were cyclized by chemical synthesis with an N- and C-terminal cysteine followed by oxidation to form a disulfide bond. However, in the present study these peptides were expressed in E. coli and cyclized with a native peptide bond between their N and C termini, which offers resistance to reducing environments, an important consideration for cyclic compounds that work intracellularly. Furthermore, these proteins lack both N and C termini and should be resistant to exoproteases and may form more stable drugs for use in serum. Novel work by Muir and co-workers (25) has demonstrated the cyclization of synthetic peptides through a peptide bond. However, chemical synthesis is currently limited to a peptide of about 100 amino acids.

Use of a bacterial expression system circumvents this limitation as demonstrated by the cyclization of thioredoxin (135 aa) and MBP (395 aa). Also, because inteins are used to generate both reactive groups, there is no need for proteases, which have been previously used to release an N-terminal cysteine for ligation reactions (18, 19). Proteases require an extra processing step, may cleave an undesired site within the target protein, and often must be removed or inactivated following treatment.

Proteins with complementary reactive groups not only can cyclize, but also may polymerize. Many proteins of structural importance, such as silks and collagen, are formed of blocks of repeating amino acid sequences. The expression of the repeating unit in monomeric form followed by in vitro polymerization may allow more rapid investigations of these compounds for the development of novel biomaterials. Furthermore, work by Zhang et al. (26, 27) have demonstrated that the interaction of small peptides can result in the formation of peptide membranes. The mechanical properties of these biomaterials may be altered or improved by polymerizing the peptides before assembly. However, an obstacle in the present study was the cyclization of thioredoxin, which then becomes unreactive to polymerization (Fig. 2). The extent of this cyclization depended on the extra amino acids added to thioredoxin. Protein structure, flexibility, and propensity to self-associate are all factors that affect whether a protein will cyclize or polymerize. Many proteins of structural importance, such as silks and collagen, are formed of blocks of repeating amino acid sequences. The expression of the repeating unit in monomeric form followed by in vitro polymerization may allow more rapid investigations of these compounds for the development of novel biomaterials. Furthermore, work by Zhang et al. (26, 27) have demonstrated that the interaction of small peptides can result in the formation of peptide membranes. The mechanical properties of these biomaterials may be altered or improved by polymerizing the peptides before assembly. However, an obstacle in the present study was the cyclization of thioredoxin, which then becomes unreactive to polymerization (Fig. 2). The extent of this cyclization depended on the extra amino acids added to thioredoxin. Protein structure, flexibility, and propensity to self-associate are all factors that affect whether a protein will cyclize or polymerize, and the investigation of these factors may allow the reaction to be biased one way or the other.

The effect of the extein residue adjacent to an intein has been observed previously (8, 17, 28). However, in the present study the C-terminal cleavage of the Ssp mini-intein (Cys → Ala) occurs in vivo with Cys-Gly following the intein (Fig. 3, lane 2), but almost no in vivo cleavage is observed with Cys-Arg (Fig. 2A, lane 1). This indicates that extein sequences at least two amino acids from the scissile peptide bond can have dramatic effects on intein activity and further demonstrates that cleavage can be modulated without changing the intein itself.
It is interesting that both the \textit{Ssp} mini-intein (Cys$^1$ → Ala) and \textit{Mxe} GyrA intein (Asn$^{198}$ → Ala) are active when separated by a linker of only 9 amino acids (see pBBP1), considering that it is possible that a short linker may constrain the folding of one or both of the inteins and inhibit the cleavage reaction. Future work should determine the minimal linker that permits the proper functioning of both inteins.

In conclusion, the TWIN system permits the facile production of bacterially expressed proteins with an N-terminal cysteine and a C-terminal thioester for use in IPL reactions. This technology will allow the investigation of large circular proteins and may allow the generation of large proteins composed of repeating units, analogous to silk proteins from arachnids or insects. In the future, controllable cleavage of Intein 1 in the TWIN system will permit the ligation of three or more protein fragments in succession.

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\textit{REFERENCES}

1. Perler, F. B., Davis, E. O., Dean, G. E., Gimble, F. S., Jack, W. E., Neff, N., Noren, C. J., Thorner, J., and Belfort, M. (1994) \textit{Nucleic Acids Res.} \textbf{22}, 1125–1127
2. Paulus, H. (1998) \textit{Chem. Soc. Rev.} \textbf{27}, 375–386
3. Perler, F. B. (1998) \textit{Cell} \textbf{92}, 1–4
4. Perler, F. B., Xu, M.-Q., and Paulus, H. (1997) \textit{Curr. Opin. Chem. Biol.} \textbf{1}, 292–299
5. Xu, M.-Q., and Perler, F. B. (1996) \textit{EMBO J.} \textbf{15}, 5146–5153
6. Chong, S., Mersha, F. B., Comb, D. G., Scott, M. E., Landry, D., Vence, I. M., Perler, F. B., Benner, J., Kucera, R. B., Hirvonen, C. A., Pelletier, J. J., Paulus, H., and Xu, M.-Q. (1997) \textit{Gene (Amst.)} \textbf{192}, 271–281
7. Evans, J. T. C., Benner, J., and Xu, M.-Q. (1998) \textit{Protein Sci.} \textbf{7}, 2256–2264
8. Mathys, S., Evans, J. T. C., Clute, I., Wu, H., Chong, S., Benner, J., Liu, X.-Q., and Xu, M.-Q. (1999) \textit{Gene (Amst.)} \textbf{231}, 1–13
9. Muir, T. W., Sondhi, D., and Cole, P. A. (1998) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{95}, 6705–6710
10. Severinov, K., and Muir, T. W. (1998) \textit{J. Biol. Chem.} \textbf{273}, 16205–16209
11. Kinsland, C., Taylor, S. V., Kelleher, N. L., McLafferty, F. W., and Begley, T. P. (1998) \textit{Protein Sci.} \textbf{7}, 1839–1842
12. Dawson, P. E., Muir, T. W., Clark-Lewis, I., and Kent, S. B. (1994) \textit{Science} \textbf{266}, 776–779
13. Tam, J. P., Lu, Y.-A., Liu, C.-F., and Shao, J. (1995) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{92}, 12485–12489
14. Evans, J. T. C., Benner, J., and Xu, M.-Q. (1999) \textit{J. Biol. Chem.} \textbf{274}, 3923–3926
15. Gimble, F. S. (1998) \textit{Chem. Biol.} \textbf{5}, R251–R256
16. Holford, M., and Muir, T. W. (1998) \textit{Structure} \textbf{6}, 945–949
17. Southworth, M. W., Amaya, K., Evans, J. T. C., Xu, M.-Q., and Perler, F. B. (1999) \textit{BioTechniques}, in press
18. Xu, R., Ayers, B., Cowburn, D., and Muir, T. W. (1999) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{96}, 388–393
19. Cotton, G. J., Ayers, B., Xu, R., and Muir, T. W. (1999) \textit{J. Am. Chem. Soc.} \textbf{121}, 1100–1101
20. Pasqualini, R., and Ruoslahti, E. (1996) \textit{Nature} \textbf{380}, 364–366
21. Yamamoto, Y., Almekhlafi, M., Katow, H., and Sotoku, S. (1995) \textit{Chem. Lett.} \textbf{1}, 11–12
22. Levi, M., Salberg, M., Ruden, U., Herlyn, D., Maruyama, H., Wigezll, H., Marke, J., and Wahren, B. (1993) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{90}, 4374–4378
23. Watanabe, T., Ito, Y., Yamada, T., Hashimoto, M., Sekine, S., and Tanaka, H. (1994) \textit{J. Bacteriol.} \textbf{176}, 4465–4472
24. Duplay, P., Bedouelle, H., Fowler, A., Zabin, I., Saurin, W., and Hofnung, M. (1984) \textit{J. Biol. Chem.} \textbf{259}, 10606–10613
25. Camarero, J. A., Cotton, G. J., Adeva, A., and Muir, T. W. (1998) \textit{J. Pept. Res.} \textbf{51}, 303–316
26. Zhang, S., Holmes, T., Lockshin, C., and Rich, A. (1993) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{90}, 3334–3338
27. Zhang, S., Holmes, T. C., DiPersio, C. M., Hynes, R. O., Su, X., and Rich, A. (1995) \textit{Biomaterials} \textbf{16}, 1385–1393
28. Chong, S., Williams, K. S., Wotkowicz, C., and Xu, M.-Q. (1998) \textit{J. Biol. Chem.} \textbf{273}, 10567–10577