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A new strategy for glycoprotein synthesis: ligation of synthetic glycopeptides with truncated proteins expressed in *E. coli* as TEV protease cleavable fusion protein

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Abstract—We report here the use of TEV protease cleavable fusion proteins to produce glycosylated bioactive peptides and proteins. Bacterial expression was utilized to produce two fusion proteins, GPRT-C37-H6 and His-tagged interleukin-2 (amino acids 6–133), which when cleaved by the tobacco etch virus NIa protease (TEV protease) to generate HIV entry inhibitor peptide C37-H6 and a truncated version of the cytokine interleukin-2, both containing N-terminal cysteines. The N-terminal cysteine containing C37-H6 and truncated interleukin-2 were then joined to a synthetic glycopeptide thioester utilizing native chemical ligation under nondenaturing and denaturing conditions, respectively. The ligations of the glycopeptide to the C37-H6 peptide and the truncated interleukin-2 protein both proceeded in high yield, though the size, and physical properties of the two polypeptides differ greatly.

1. Introduction

The activity of proteins and peptides can be affected by glycosylation in a wide variety of manners. Glycosylation can increase the serum half-life of proteins, as demonstrated by increased serum half-life of fully sialylated erythropoietin when compared to erythropoietin with lower levels of sialylation.[1] Also, glycosylation can enhance crossing of the blood–brain barrier, as has been observed with nonnatural glycosylation of enkephalin peptides.[2,3] In addition, glycosylation has been shown to alter bioactivity and reduce susceptibility to proteases in glycosylation studies of peptides such as the *Saccharomyces cerevisiae* α-mating factor.[4] Though glycosylation can have several desirable effects, it is very difficult to incorporate glycosylation into peptides and proteins produced in bacteria. We have recently reported the use of tobacco etch virus NIa protease (TEV) cleavable fusion proteins in the incorporation of synthetic labels onto the N-terminus of bacterially expressed proteins.[5] Here we investigate the use of TEV cleavable fusion proteins in the glycosylation of bioactive peptides and proteins produced in bacteria.

2. Results and discussion

We chose C37-H6, a peptide derived from the HIV gp41, and a truncated form of human interleukin-2 as models for glycosylation of bioactive peptides and proteins with TEV protease cleavable fusion proteins. The peptide C37-H6 is an HIV entry inhibitor peptide with nanomolar activity,[6] and human interleukin-2 is a T-cell growth factor that is used therapeutically to treat renal cell carcinoma and metastatic melanoma.[7] In addition, both C37-H6 and interleukin-2 are hydrophobic, and we reasoned that addition of hydrophilic sugars to such hydrophobic polypeptides would have maximum effect on the biophysical behavior of those hydrophobic polypeptides, that would hopefully translate into either useful changes in the physical properties of the polypeptides or increased bioactivity.

Fusion proteins that contain TEV protease cleavable linkers were constructed for both C37-H6 and a truncated form of human interleukin-2. For the HIV entry inhibitor peptide C37-H6, a bacterial protein, xanthine-guanine phosphoriboysyltransferase (GPRT),

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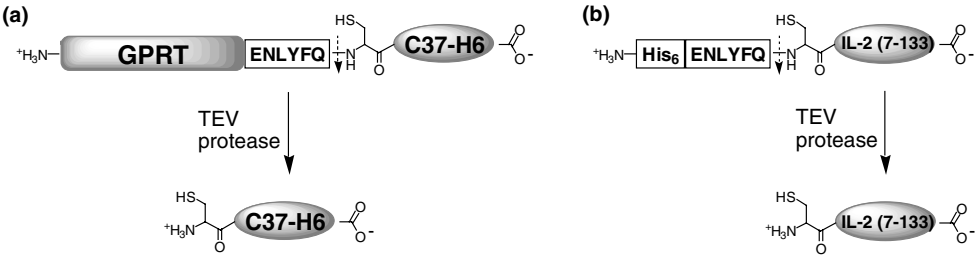


Figure 1. (a) TEV protease cleavage of the GPRT-C37-H6 fusion protein to give the C37-H6 HIV entry inhibitor peptide with an N-terminal cysteine. (b) TEV protease cleavage of the His-tagged truncated interleukin-2 to yield truncated interleukin-2 (6–133) with a Ser→Cys mutation at residue 6.

was fused to the N-terminus of the peptide with a TEV protease cleavable linker between the protein and the peptide (Fig. 1). The GPRT fusion partner served to stabilize the peptide during expression of the fusion protein in bacteria. GPRT is a small protein of around 153 amino acids that folds into a stable tetrameric structure. Rather than use HIV viral RNA sequences to construct the C37-H6 peptide, DNA for the C37-H6 peptide was constructed from synthetic DNA oligonucleotides with PCR using codons optimized for *Escherichia coli* expression. Overexpression of the GPRT-C37-H6 fusion protein in *E. coli* resulted in approximately 21% of total cellular protein being the GPRT-C37-H6 fusion protein (Fig. 2). A truncated form of human interleukin-2 was produced by fusing a TEV protease cleavable His-tag to the N-terminus of amino acids 6–133 of the interleukin-2 protein sequence with a Ser to Cys mutation at residue 6 (Fig. 1). Expression of the truncated His-tagged interleukin-2 resulted in inclusion bodies that were purified under denaturing conditions (Fig. 2). Oxidation and refolding of the purified protein resulted in water soluble, His-tagged, truncated interleukin-2.

TEV protease cleavage of both the GPRT-C37-H6 and His-tagged truncated interleukin-2 fusion proteins produced the desired polypeptides with N-terminal cysteines, with the correct molecular weights confirmed by MALDI-TOF mass spectrometry. The C37-H6 peptide and truncated interleukin-2 were then used in native chemical ligation reactions[8] to join them to a test glycopeptide thioester, H-Asn(GlcNAc)-Gly-Gly-thioester (Fig. 3).

The C37-H6 peptide is water soluble, and so it was possible to ligate it to the H-Asn(GlcNAc)-Gly-Gly-thioester under nondenaturing conditions. Ligation of the C37-H6 peptide to the glycopeptide thioester gave a high yield of glycosylated C37-H6 (>95%) as observed by MALDI-TOF mass spectrometry (Fig. 4).

In contrast to the C37-H6 peptide, strongly denaturing conditions were required to ligate interleukin-2 to the test glycopeptide. The strongly denaturing conditions were required because interleukin-2 is an extremely hydrophobic protein that has a tendency to aggregate and precipitate when its single, structurally important disulfide bond is broken. Since native chemical ligation requires strongly reducing conditions to prevent oxidation of protein-based cysteines, native chemical ligation of interleukin-2 had to be conducted in strongly denaturing conditions (≈7 M guanidine hydrochloride) to keep the truncated form of interleukin-2 in solution during ligation reactions. When the truncated form of interleukin-2 was ligated to the H-Asn(GlcNAc)-Gly-

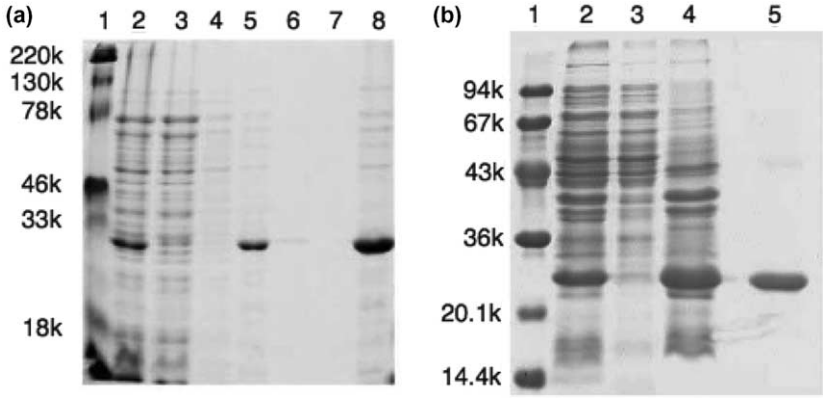


Figure 2. (a) Purification gel of GPRT-C37-H6. (1) Molecular weight markers, (2) cell lysate, (3) Ni–NTA column flow through, (4) column wash, (5) elution (fraction 1), (6) elution (fraction 2), (7) elution (fraction 3), (8) purified GPRT-C37-H6 after dialysis. (b) Purification gel of His-tagged IL-2 fragment. (1) Molecular weight markers, (2) whole cell extract, (3) cell lysate, (4) inclusion bodies, (5) purified His-tagged IL-2 after Ni–NTA column.

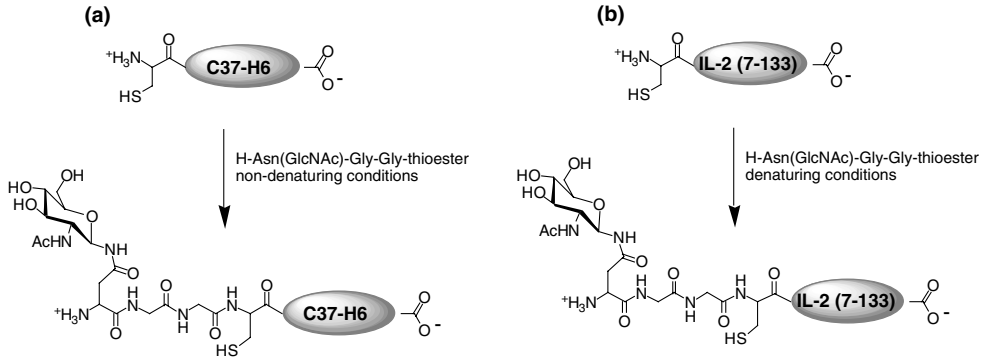


Figure 3. (a) Ligation of C37-H6 to the H-Asn(GlcNAc)-Gly-Gly-thioester under nondenaturing conditions. (b) Ligation of the truncated interleukin-2 to the H-Asn(GlcNAc)-Gly-Gly-thioester under denaturing conditions.

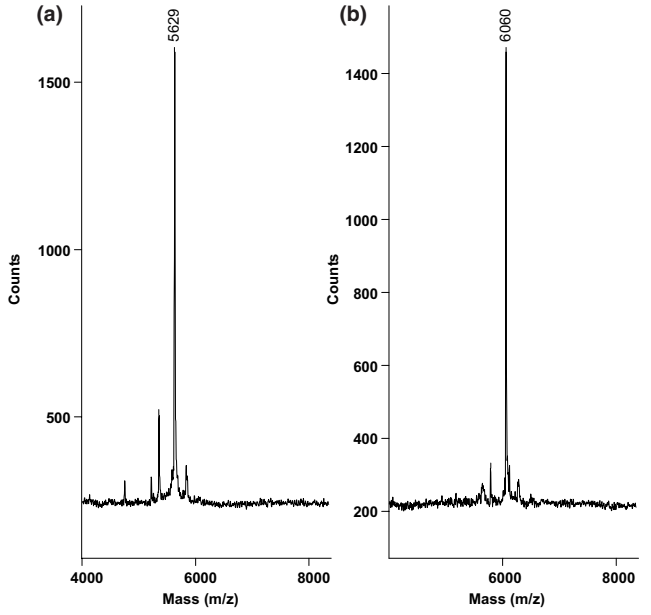


Figure 4. MALDI-TOF mass spectra of (a) HIV entry inhibitor peptide C37-H6, calcd mass 5628 and (b) HIV entry inhibitor peptide C37-H6 ligated to the model glycopeptide (H-Asn(GlcNAc)-Gly-Gly), calcd mass 6059.

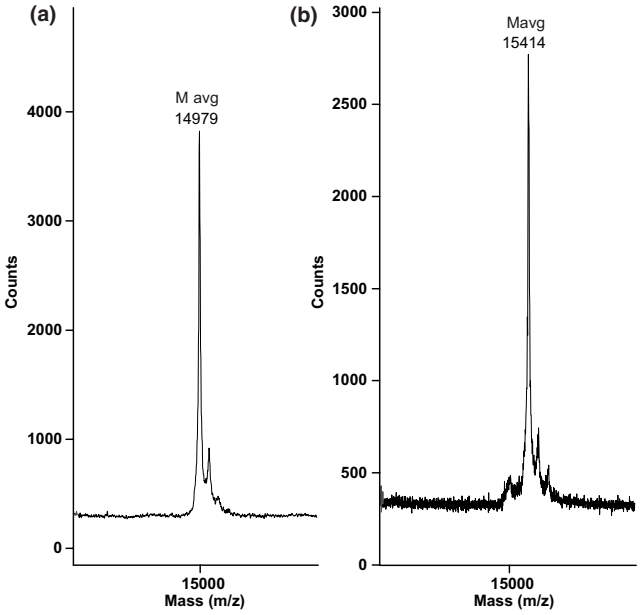


Figure 5. MALDI-TOF mass spectra of (a) truncated interleukin-2, calcd mass 14,975 and (b) truncated interleukin-2 ligated to the model glycopeptide (H-Asn(GlcNAc)-Gly-Gly), calcd mass 15,406.

Gly-thioester under strongly denaturing conditions, a high yield of glycosylated interleukin-2 (>95%) was observed (Fig. 5).

3. Conclusion

The use of TEV protease cleavable fusion proteins to glycosylate bioactive peptides and proteins is an efficient method for incorporating glycosylation into bacterially expressed proteins. As demonstrated here, this approach can be used for both water-soluble peptides, such as C37-H6, and also very hydrophobic proteins that are not soluble under certain conditions, such as interleukin-2. For production of bioactive peptides, fusion partners can be chosen to reduce degradation of peptides expressed in bacteria, and then removed with a TEV protease cleavage step, as demonstrated with the GPRT-C37-H6 fusion protein produced in this paper. Affinity tags can be incorporated within the fusion protein to allow affinity purification, and then removed during the TEV protease cleavage step so that the affinity tags do not affect the function of the bioactive protein, as demonstrated with the truncated interleukin-2 produced in this paper. In addition, N-terminal cysteines are reactive chemical moieties that can form nonreactive adducts with aldehydes, and masking of N-terminal cysteines until their use during native chemical ligation reactions prevents the formation of these aldehyde adducts. Ligation of both the C37-H6 HIV entry inhibitor peptide and the truncated interleukin-2 protein to the test glycopeptide proceeded smoothly, and it should be possible to incorporate a wide variety of glycosylation onto bioactive peptides and proteins using these approaches. Tests are currently underway for evaluating the effect of glycosylation on C37-H6 and interleukin-2.

4. Experimental

4.1. General

Chemicals were purchased from Aldrich and Sigma. Protected amino acids were obtained from Novabiochem and Bachem. Nucleic acid manipulation were done according to standard procedures.[9] Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. *Pfx* DNA polymerase was purchased from Invitrogen. Primers were ordered from MWG Biotech AG. UV assays were performed on a Cary 3 Bio UV–Vis spectrophotometer. NMR spectra were recorded on a Brucker AMX 400 NMR spectrometer. MALDI-TOF mass spectrometry of protein samples were conducted with an Applied Biosystems Voyager-DE. DNA sequencing was performed on an ABI 377A automated sequencer. The reactions were performed using thermal cycle sequencing conditions with fluorescent labeled terminators. Strains, plasmids, and primers used in this study are shown (Table 1).

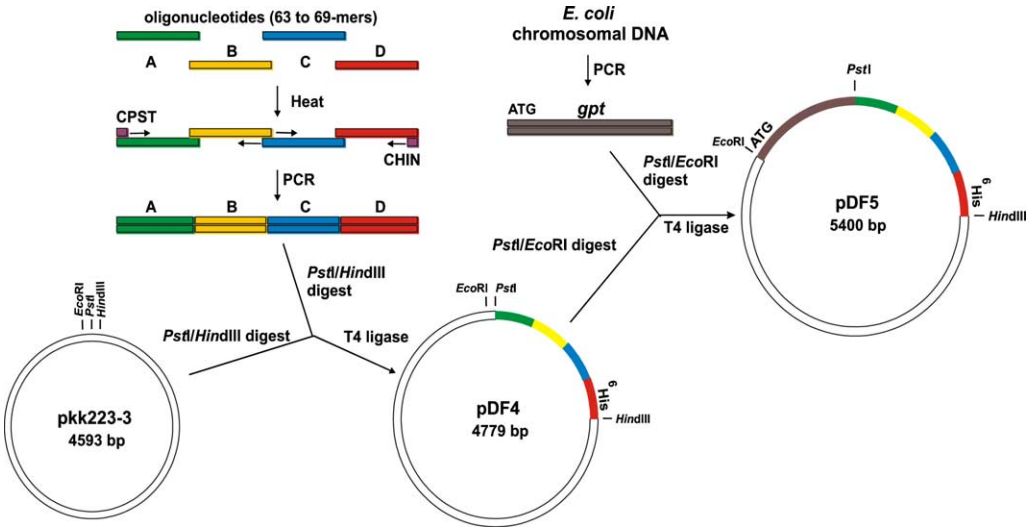
Protein concentrations were determined by the Bradford procedure (Biorad, Hercules, CA), using BSA as the calibration standard. *E. coli* strains were grown on Luria-Bertani (LB) medium.[9] Ampicillin (amp) was added at a concentration of $100\,mg\,\mu L^{-1}$ when required. For long-term storage, cells in the mid-exponential phase of growth were harvested and shock-frozen in 50% glycerol suspension at −80 °C. Protein solutions were frozen in liquid nitrogen and stored at 78 °C.

4.2. Cloning of GPRT-C37-H6

The gene encoding GPRT, *gpt*, was amplified from chromosomal DNA of *E. coli* JM109. The concentration of chromosomal DNA was $0.1\,ng\,\mu L^{-1}$. *Gpt* was generated by using $0.5\,pmol\,\mu L^{-1}$ of primers GECO and GPST. After an initial denaturation step of 4 min at 94 °C, 34 cycles of 30 s at 94 °C, 90 s at 55 °C, and 90 s at 72 °C were performed. A final extension step of 5 min at 72 °C and cooling to 4 °C completed the reaction. *Gpt* was obtained as a single product. The gene for C37 was amplified from overlapping fragments A, B, C, and D ($0.05\,pmol\,\mu L^{-1}$ each) using $0.5\,pmol\,\mu L^{-1}$ of terminating primers CPST and CHIN. After an initial denaturation step of 4 min at 94 °C, 10 cycles of 30 s at 94 °C, 90 s at 55 °C, and 60 s at 72 °C were performed. A final extension step of 3 min at 72 °C and cooling to 4 °C completed the reaction. The gene for C37 was obtained in mixture with unspecific smaller reassembled products and was not further purified (Scheme 1).

Table 1. Strains, plasmids, and primers used in this study

	Relevant characteristic(s)	Origin and refs
Escherichia coli strains		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15 Tn* 10 (Tet^r)], cloning strain	Ref. 10
DH5αF′	F$^-$ *endA1 hsdR17* ($r_k^-m_k^+$) *recA1 supE44 thi-1* Δ*(lacZYA-argF)U169 ϕ80lac*ZΔM15, cloning strain	Ref. 11
JM109	e14$^-$(McrA$^-$) *recA1 endA1 gyrA96 thi-1 hsdR17* ($r_K^-m_K^+$) *supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*ZΔM15*], expression strain	Ref. 12
Plasmids		
pkk233-3	amp^R, cloning vector	Ref. 13
pDF4	amp^R (C37 obtained from fragments A–D and primers CPST and CHIN cloned into pkk233-3 cut with *Pst*I and *Hin*dIII)	This study
pDF5	amp^R (*gpt* obtained with primers GECO and GPST cloned into pDF4 cut with *Pst*I and *Eco*RI)	This study
pLW46	amp^R, tet^R (vector containing the gene for human interleukin-2)	Ref. 14
pTrcHisB	amp^R, cloning vector	
pTrcSal1	amp^R (linker obtained from annealing of L1 and L2 cloned into pTrcHisB cut with *Kpn*I and *Bgl*II)	This study
pCys6IL2	amp^R (gene encoding for interleukin-2 obtained with primers ISAL and IECO cloned into pTrcSal1 cut with *Sal*I and *Eco*RI)	This study
Primers		
GECO	(*Eco*RI) 5′-CCG CGC GAA TTC ATG AGC GAA AAA TAC ATC GTC ACC-3′	This study
GPST	(*Pst*I) 5′-CCG GCG CTG CAG GCG ACC GGA GAT TGG CGG GAC GAA-3′	This study
CPST	(*Pst*I) 5′-GGG GCG CTG CAG GGT GAA AAC C-3′	This study
CHIN	(*Hin*dIII) 5′-GGG CCC AAG CTT TTA GTG GTG-3′	This study
ISAL	(*Sal*I) 5′-CCG CGC GTC GAC GAA AAC CTG TAT TTT CAG TGC ACA AAG AAA ACA CAG CTA-3′	This study
IECO	(*Eco*RI) 5′-CCG GCG GAA TTC TCA AGT CAG TGT TGA GAT GAT GCT-3′	This study
L1	5′-GAT CTG ATT ACG ATA TCC CAA CGA CCG TCG ACG CTG GTA C-3′	This study
L2	5′-CAG CGT CGA CGG TCG TTG GGA TAT CGT AAT CA-3′	This study
A	(*Pst*I) 5′-GGG GCG CTG CAG GGT GAA AAC CTG TAT TTT CAG TGC GGT CAC ACC ACC TGG ATG GAA TGG GAC-3′	This study
B	5′-GAT CAG GGA GTG GAT CAG GGA GGT GTA GTT GTT GAT TTC ACG GTC CCA TTC CAT CCA GGT GGT-3′	This study
C	5′-TCC CTG ATC CAC TCC CTG ATC GAA GAA TCC CAG AAC CAG CAG GAA AAA AAC GAA CAG GAA CTG CTG GGT-3′	This study
D	(*Hin*dIII) 5′-GGG CCC AAG CTT TTA GTG GTG GTG GTG GTG GTG ACC CAG CAG TTC CTG TTC GTT TTT TTC CTG CTG-3′	This study



Scheme 1. Construction of a GPRT-C37-H6 expression plasmid.

Plasmid pkk233-3 (Amersham Pharmacia Biotech, Foster City, CA) and the gene for C37 were cut with *Pst*I and *Hin*dIII and afterwards ligated was done using T4 DNA ligase. Transformation of *E. coli* XL1-Blue with the resulting pDF4 gave the recombinant strain, which shows ampicillin resistance. Correct ligation and transformation were verified by restriction site analyses. The PCR product of *gpt* was restricted with *Eco*RI and *Pst*I and analogously cloned into the vector pDF4 to give vector pDF5. Transformation of *E. coli* XL1-Blue with pDF5 followed by restriction analysis, verified the correct insertion of *gpt*.

4.3. Production and purification of GPRT-C37-H6

A volume of 1 L of LB medium (amp) was inoculated with 10 mL of pre-culture (LB medium, amp, 37 °C, 200 rpm, 14 h) or from plate (single colony). The culture was cultivated at 200 rpm and 24 or 37 °C. Induction was done at an OD600 of 0.6 using 200 μM IPTG. Further cultivation was done at 200 rpm and either 4, 24, or 37 °C.

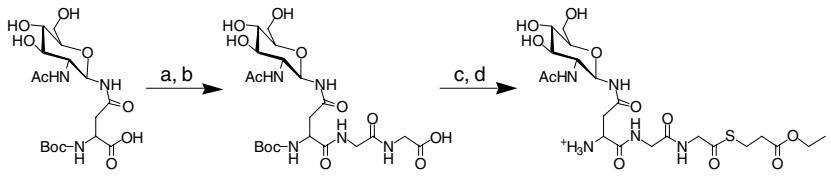
For protein purification cell pellets were re-suspended in 25 mL of 50 mM potassium phosphate (KP) buffer (pH 7.5, chilled on ice), containing 5 mM β-mercaptoethanol and 300 mM NaCl, and was lyzed by passing through a French Press compressed to 1500 psi and then released to ambient pressure. The process was repeated three times. Cell debris was pelleted by centrifugation (12,000*g*, 4 °C, 1 h). The supernatant was filtered through a cellular acetate membrane filter (0.2 μm), and loaded onto a Ni^{2+}-NTA-agarose (2.5 mL bed volume, pre-equilibrated with cell re-suspension buffer). The column was washed with 20 mL of 50 mM KP buffer (pH 7.5), containing 5 mM β-mercaptoethanol, 300 mM NaCl, 5% glycerol, and 10 mM imidazole, followed by 20 mL of 50 mM KP buffer (pH 7.5), containing 5 mM β-mercaptoethanol, and 10 mM imidazole. Bound protein was eluted in 50 mM KP buffer (pH 7.5), containing 5 mM β-mercaptoethanol and 250 mM imidazole. The solution was dialyzed extensively against 50 mM KP (pH 7.5), containing 5 mM β-mercaptoethanol at 4 °C. Eluted proteins were analyzed with SDS-PAGE and were found to be >90% pure in any of three experiments.

4.4. Cloning of His-tagged interleukin-2 (6–133)

Construction of an affinity-tagged interleukin-2 expression plasmid was done in analogy to the construction of the overexpression system for GPRT-C37-H6. Plasmid pTrcHisB (Invitrogen) was double digested with *Kpn*I and *Bgl*II restriction enzymes and the linearized product was purified. A synthetic double stranded fragment of DNA was formed by annealing the synthetic oligonucleotides L1 and L2. The resulting double stranded fragment was phosphorylated using T4 polynucleotide kinase and ligated into pTRcHisB using T4 DNA ligase to afford pTrcSal1. *E. coli* XL1-Blue was transformed with the ligation product and the correct insert was confirmed by DNA sequencing. A DNA fragment encoding a TEV protease cleavage site with a cysteine mutation at the P1′ position fused to a fragment of human interleukin-2 (DNA encoding amino acids 7–133) was produce by PCR using the primers ISAL and IECO and interleukin-2 encoding plasmid pLW46 (ATCC 39452) as template. The PCR fragment was inserted into pTrcSal1 using *Sal*I and *Eco*RI restriction sites and T4 DNA ligase to afford plasmid pCys6IL2. Correct insertion of the PCR fragment DNA was verified by DNA sequencing.

4.5. Production and purification of His-tagged interleukin-2 (6–133)

The affinity-tagged interleukin-2 fusion protein was expressed from pCys6IL2 in *E. coli* DH5αF′ cells as inclusion bodies. *E. coli* DH5αF′ was transformed with pCys6IL2. The resulting strain DH5αF′/pCys6IL2 was inoculated in 10 mL LB medium (amp), containing 0.4% glucose at 37 °C and 200 rpm for 14 h. A volume of 1 L LB medium (amp) was inoculated with this preculture and incubated (37 °C, 200 rpm, 14 h). Induction was done at OD600 of 0.4 using 200 μM IPTG and the



Scheme 2. Synthesis of H-Asn(GlcNAc)-Gly-Gly-thioester tripeptide. (a) H-Gly-Gly-OBn, DIC, HOBt, NMM; (b) MeOH, 10% Pd on C, H_2; (c) ethyl 3-mercaptopropionate, DIC, HOBt; (d) trifluoroacetic acid/water (95:5).

culture incubated for another 6 h (37 °C, 200 rpm). Cells were harvested by centrifugation (9000*g*, 4 °C, 15 min), resuspended in 50 mM sodium phosphate (SD) buffer (pH 8.0, 20 mM β-mercaptoethanol) and passed three times through a French Press as described above. Cell debris and inclusion bodies were pelleted by centrifugation (12,000*g*, 4 °C, 30 min) and re-suspended in 50 mM SD buffer (pH 8.0, 10 mM β-mercaptoethanol, 7 M guanidine hydrochloride) by vigorous stirring for 1 h. The re-suspended inclusion body pellet was removed by centrifugation (12,000*g*, 4 °C, 1 h) and by filtration through a 0.2 μm syringe filter prior to loading on a Ni^{2+}-NTA-agarose (2.5 mL bed volume, pre-equilibrated with cell re-suspension buffer). Washing and elution of the protein was done as described for the purification of GPRT-C37-H6. Dithiothreitol (DTT) was added to the purified protein solution to a concentration of 10 mM and the protein solution was dialyzed against 50 mM SD buffer (pH 7.0). Precipitated, reduced interleukin-2 was lyophilized and dissolved in 50 mM SD buffer (pH 8.0, 7 M guanidine hydrochloride) to a concentration of approx. 1 mg (protein) mL^{-1}. The protein solution was gently stirred under an air atmosphere for 3 days and then dialyzed against several changes of 50 mM SD buffer (pH 8.0) with 1000 MWCO dialysis tubing. A small amount of precipitated protein was removed by centrifugation. The resulting protein solution contains oxidized, refolded His-tagged interleukin-2 suitable for TEV cleavage reaction.

4.6. TEV protease cleavage of GPRT-37-H6

A solution of 1 mg of GPRT-C37-H6 in 0.85 mL of buffer was placed into a 1000 MWCO dialysis bag and 100 units of TEV protease were added. The dialysis bag was placed into 1 L of 50 mM SD buffer (pH 7.8). The TEV protease cleavage reaction was initiated by adding β-mercaptoethanol to the dialysis buffer to a final concentration of 1 mM. The reaction was gently stirred and incubated at 24 °C for approx. 24 h. The cleavage reaction was analyzed by SDS-PAGE and MALDI-TOF mass spectrometry to confirm formation of the correctly cleaved C37-H6 peptide: calcd 5628, found 5629.

4.7. TEV protease cleavage of His-tagged interleukin-2 (6–133)

TEV protease cleavage of the His-tagged interleukin-2 (oxidized and refolded) was done under dialysis conditions to minimize thiazolidine formation. Ten milligrams of His-tagged-IL2 was placed in a 1000 MWCO dialysis bag and 1000 units TEV protease was added to this solution. The dialysis bag was placed in 50 mM SD buffer (pH 7.8). After initiation of the reaction by adding β-mercaptoethanol to the dialysis buffer to a final concentration of 1 mM, the reaction was gently stirred and incubated at ambient temperature for approx. 24 h. The cleavage reaction was analyzed by SDS-PAGE and MALDI-MS to confirm the formation of the cleaved interleukin-2 fragment.

4.8. Synthesis of H-Asn(GlcNAc)-Gly-Gly-thioester

The synthesis of H-Asn(GlcNAc)-Gly-Gly-thioester tripeptide is shown Scheme 2.

4.8.1. Synthesis of 1 (Boc-Asn(GlcNAc)-Gly-Gly-OH). Boc-Asn(GlcNAc)-OH (0.91 g, 2.0 mmol) was activated with 2 equiv of diisopropylcarbodiimide (0.63 mL, 4.0 mmol) and 2 equiv of HOBt (0.61 g, 4.0 mmol) in DMF (35 mL) by stirring for 15 min. Then 1.1 equiv of H-Gly-Gly-OBn *p*-tosylate salt (0.87 g, 2.2 mmol) and 4 equiv of *N*-methyl morpholine (0.88 mL, 8.0 mmol) were added to the reaction. The reaction was stirred at 24 °C for 4.5 h and then concentrated in vacuo. The residue was dissolved in a minimum amount of methanol and precipitated with diethyl ether. The precipitate was collected by filtration and washed with diethyl ether. The resulting precipitate was dissolved in methanol (75 mL) and 10% palladium on carbon (50 mg) was added to the solution. This mixture was placed under a hydrogen atmosphere (1 atm pressure) and stirred for 5 h. All nonsoluble material was removed by filtration through Celite and the resulting filtrate was concentrated in vacuo. The resulting residue was purified by flash chromatography using ethyl acetate/methanol/water (70:20:10) as eluent to give 0.75 g of the white solid **1**. ^{1}H NMR (400 MHz, CD_3OD) δ 4.99 (d, $^3J_{(H,H)}$ = 9.7 Hz, 1H), 4.45 (t, $^3J_{(H,H)}$ = 5.7 Hz, 1H), 4.01–3.84 (m, 5H), 3.76 (t, $^3J_{(H,H)}$ = 10.1 Hz, 1H), 3.67 (dd, $^3J_{(H,H)}$ = 11.6, 4.6 Hz, 1H), 3.53 (t, $^3J_{(H,H)}$ = 9.1 Hz, 1H), 3.39–3.30 (m, 2H), 2.72 (d, $^3J_{(H,H)}$ = 5.9 Hz, 2H), 1.99 (s, 3H, $COCH_3$), 1.45 (s, 9H, $C(CH_3)_3$). MALDI-FTMS: calcd for $C_{21}H_{35}N_5O_{12}Na^+$ 572.2180, found 572.2175.

4.8.2. Synthesis of 2 (H-Asn(GlcNAc)-Gly-Gly-thioester). Boc-Asn(GlcNAc)-Gly-Gly-OH (**1**) (0.35 g, 0.65 mmol) was placed into a flask and dissolved in DMF (25 mL). Diisopropylcarbodiimide (4 equiv, 0.41 mL, 2.6 mmol), HOBt (2 equiv, 0.20 g, 1.3 mmol), and ethyl 3-mercaptopropionate (5 equiv, 0.42 mL, 3.2 mmol) were added to

the solution. The mixture was stirred at 24 °C for 24 h, and the solvent was removed under reduced pressure. The resulting residue was dissolved in a minimum amount of methanol and precipitated with diethyl ether. The precipitate was collected by filtration and washed with diethyl ether. The precipitate was dissolved in a 10 mL solution of trifluoroacetic acid/water (95:5) and stirred for 15 min. The solvent was removed in vacuo, and the residue was azeotroped three times with toluene. The resulting residue was purified by flash chromatography using a mixture of ethyl acetate/methanol/water (70:20:10) as eluent to yield 0.21 g of the glycosylated tripeptide thioester (**2**) as a white solid. ^{1}H NMR (400 MHz, D_2O) δ 5.08 (d, $^3J_{(H,H)} = 9.7$ Hz, 1H), 4.21–4.15 (m, 4H), 4.06 (d, $^2J_{(H,H)} = 17$ Hz, 1H), 4.01 (d, $^2J_{(H,H)} = 17$ Hz, 1H), 3.91–3.74 (m, 4H), 3.63 (t, $^3J_{(H,H)} = 9.1$, 1H), 3.55–3.46 (m, 2H), 3.18 (t, $^3J_{(H,H)} = 6.8$ Hz, 2H, CO_2CH_2), 2.79–2.62 (m, 4H), 2.03 (s, 3H, $COCH_3$), 1.27 (t, $^3J_{(H,H)} = 7.2$, 3H, terminal CH_3); ^{13}C NMR δ 202.00 (COS), 178.01, 176.32, 175.89, 175.02, 173.88, 79.86, 79.20, 75.84, 71.08, 63.61, 62.11, 55.82, 53.04, 50.54, 44.03, 41.52, 35.41, 25.03, 23.65, 14.92 (terminal CH_3). MALDI-FTMS: calcd for $C_{21}H_{35}N_5O_{11}SNa^+$ 588.1946, found 588.1965.

4.9. Ligation of H-Asn(GlcNAc)-Gly-Gly-thioester to C37-H6

The cleaved C37-H6 peptide (0.6 mg) in 0.5 mL of 50 mM SD buffer (pH 7.8) was placed into a 2 mL Eppendorf tube. β-Mercaptoethanesulfonic acid (29 mM final concentration) and a 37 mM solution of the H-Asn(GlcNAc)-Gly-Gly-thioester (3.8 mM final concentration). This mixture was incubated at 24 °C for 20 h, desalted, and purified by reverse phase HPLC. The resulting ligated glycosylated interleukin-2 was analyzed by MALDI-TOF mass spectrometry: calcd 6059, found 6060.

4.10. Ligation of H-Asn(GlcNAc)-Gly-Gly-thioester to interleukin-2 (6–133)

Native chemical ligation was used to link the cleaved interleukin-2 fragment to H-Asn(GlcNAc)-Gly-Gly-thioester. The TEV protease cleaved interleukin-2 fragment (1 mg mL^{-1}, 3 mL buffer solution) was precipitated by adding solid ammonium sulfate and gentle mixing until the solution had a saturation of ammonium sulfate of 90%. The precipitated ammonium sulfate pellet was collected by centrifugation and dissolved in 0.44 mL of a solution consisting of 50 mM SD buffer (pH 7.6), 6.8 M guanidine hydrochloride, 30 mM 2-mercaptoethanesulfonic acid, and 5 mM of H-Asn(GlcNAc)-Gly-Gly-thioester. The mixture was incubated at 24 °C for 24 h. The protein solution was desalted and purified by reverse phase HPLC. The formation of the glycosylated interleukin-2 is observed by MALDI-TOF mass spectrometry: calcd 15406, found 15414.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2004.06.047.

References and notes

1. Varki, A.; Cummings, R.; Esko, J.; Freeze, H.; Marth, J.; Hart, G. *The Essentials of Glycobiology*; Cold Spring Harbor Laboratory: New York, 1999.
2. Bilsky, E. J.; Egleton, R. D.; Mitchell, S. A.; Palian, M. M.; Davis, P.; Huber, J. D.; Jones, H.; Yamamura, H. I.; Janders, J.; Davis, T. P.; Porreca, F.; Hruby, V. J.; Polt, R. *J. Med. Chem.* **2000**, *43*, 2586.
3. Egleton, R. D.; Mitchell, S. A.; Huber, J. D.; Palian, M. M.; Polt, R.; Davis, T. P. *J. Pharmacol. Exp. Ther.* **2001**, *299*, 967.
4. Saskiawan, I.; Mizuno, M.; Inazu, T.; Haneda, K.; Harashima, S.; Kumagai, H.; Yamamoto, K. *Arch. Biochem. Biophys.* **2002**, *406*, 127.
5. Tolbert, T. J.; Wong, C.-H. *Angew. Chem., Int. Ed.* **2002**, *41*, 2171.
6. Root, M. J.; Kay, M. S.; Kim, P. S. *Science* **2001**, *291*, 884.
7. Atkins, M. B. *Semin. Oncol.* **2002**, *29*, 12.
8. Dawson, P. E.; Kent, S. B. *Annu. Rev. Biochem.* **2000**, *69*, 923.
9. Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory: New York, 1989.
10. Bullock, W. O.; Fernandez, J. M.; Short, J. M. *Biotechniques* **1987**, *5*, 376.
11. Hanahan, D. *J. Mol. Biol.* **1983**, *166*, 557.
12. Yanisch-Perron, C.; Vieira, J.; Messing, J. *Gene* **1985**, *33*, 103.
13. Nock, S.; Lechler, A.; Ribeiro, S.; Kreutzer, R. *Biotechniques* **1995**, *18*, 608.
14. Wang, A.; Lu, S. D.; Mark, D. F. *Science* **1984**, *224*, 1431.