SUPPORTING INFORMATION

Role of a cytoplasmic dual-function glycosyltransferase in O_2-regulation of development in *Dictyostelium*

Zhuo A. Wang[1], Hanke van der Wel[1], Yusof Vohra[2], Altan Ercan[1,3], Christa L. Feasley[1], Therese Buskas[2], Geert-Jan Boons[2], and Christopher M. West[1]

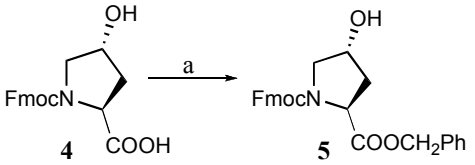
[1]Dept. of Biochemistry & Molecular Biology and Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104 USA; [2]Dept. of Chemistry and Complex Carbohydrate Research Center, 315 Riverbend Road, University of Georgia, Athens, GA 30602 USA.

Synthesis of Glycopeptide 1 and peptides 2 and 3.

Reagents and general methods.

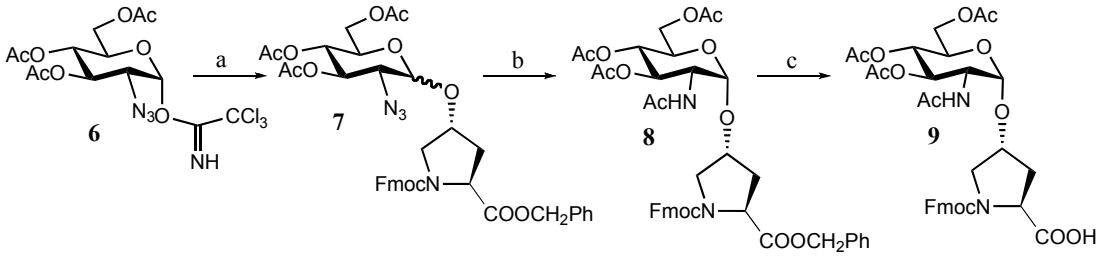
All solvents employed were reagent grade. CH_2Cl_2, methanol and diethyl ether were distilled from CaH_2 prior to use in reactions. All the starting materials were kept *in vacuo* with P_2O_5 prior to use. Chemicals were purchased from Aldrich and Fluka and used without further purification. All Fmoc-amino acids, Rink Amide AM resin and *O*-(7-aza-benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) were purchased from NovaBioChem. *N*,*N*-dimethylformamide (DMF) was obtained from EM Science, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylhexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBt), and *N*-methylpyrrolidone (NMP) from Applied Biosystems. Succinimidyl 3-(bromoacetamido)propionate (SBAP), mariculture keyhole limpet hemocyanin (mcKLH), and maleimide activated BSA were purchased from Pierce Endogen. Column chromatography was performed on silica gel G60 (SiliCycle, 60-200μm 60 Å), reactions were monitored by TLC on Silicagel 60 F_{254} (EMD Chemicals Inc.). The compounds were detected by examination under the UV light and visualized by charring with 10% sulfuric acid in methanol or cerium ammonium molybdate in 20% aq. sulfuric acid. Solvents were removed under reduced pressure at ≤ 30 °C. ^{1}H-NMR and HSQC spectra were recorded in $CDCl_3$ at 500 MHz on a Varian Inova spectrometer with tetramethylsilane as internal standard, unless otherwise stated. High-resolution mass spectra were obtained by using MALDI-ToF (Applied Biosystems 4700 Proteomics Analyzer) with 2,5-dihydroxybenzoic acid as matrix and the internal standards ultramark 1621 and PEG.

***N*-α-(9-Fluorenylmethyloxycarbonyl)-L-*trans*-4-hydroxyproline benzyl ester (5):** *N*-α-(9-Fluorenylmethyloxycarbonyl)-L-*trans*-4-hydroxyproline **(4)** (1.0 g, 2.83 mmol) was taken as a suspension in methanol (20 ml). $CsCO_3$ (510 mg, 1.56 mmol) was added at room temperature followed by stirring at the same temperature for 1 h. The reaction mass was filtered and the filtrate was concentrated *in vacuo* to afford a white amorphous solid. The solid was taken in DMF (10 ml), followed by addition of benzyl bromide (0.40 ml, 3.39 mmol) and stirred at room temperature for 2 h. The reaction mass was poured into water (100 ml) with stirring. The compound was extracted with EtOAc (50 ml), washed with water (50 ml), and brine (25 ml). The



Scheme S1. Preparation of Hydroxy proline ester. *Reagents and Conditions:* **a**) i. CsCO3, MeOH, ii. BnBr, DMF, quant yield.

organic layer was dried ($MgSO_4$), filtered and the filtrate was concentrate *in vacuo*. The residue was purified by silica gel column chromatography (EtOAc:Hexanes, 1/1, v/v) to afford compound **5** (1.5 g, 99%), as a white solid (exists as a mixture of rotational isomers). Analytical data for **5**: R_f = 0.60 (EtOAc:Hexanes:AcOH, 60:40:2, v/v/v); ^{1}H-NMR (500 MHz, $CDCl_3$) : δ = 7.80-7.25 (m, 26H, aromatic), 5.27-5.17 (q, 2H, C*H*$_2$Ph-R_1), 5.16-5.07 (q, 2H, C*H*$_2$Ph-R_2), 4.63 (t, 1H, *J* = 7.8 Hz, H-αR_1), 4.58 (t, *J* = 7.9 Hz, H-αR_2), 4.54 (bm, 1H, H-γR_1), 4.50 (bm, 1H, H-γR_2), 4.47 (m, 1H, C*H*H-FmocR_1), 4.41 (m, 1H, C*H*H-FmocR_1), 4.37 (m, 1H, C*H*H-FmocR_2), 4.29 (m, 2H, C*H*H-FmocR_2, C*H*-FmocR_1), 4.02 (t, 1H, *J* = 6.9 Hz, C*H*-FmocR_2), 3.78 (m, 1H, H-δaR_1), 3.70 (m, 2H, H-δaR_2, H-δbR_2), 3.59 (bd, 1H, H-δbR_1), 2.42 (m, 1H, H-βaR_1), 2.38 (m, 1H, HβaR_2), 2.15 (m, 2H, H-βbR_1, H-βbR_2) ppm; ^{13}C from HSQC (75 MHz, $CDCl_3$) : δ = 172.56, 172.50, 155.26, 154.96, 144.38, 144.27, 144.04, 143.78, 141.56, 141.53, 141.42, 135.76, 135.57, 130.15, 128.78, 128.64, 128.53, 128.34, 127.93, 127.87, 127.31, 125.43, 125.36, 125.21, 120.19, 120.15, 70.38, 69.55, 67.99, 67.83, 67.24, 67.16, 58.28, 57.98, 55.55, 54.87, 47.42, 47.34, 39.55, 38.59 ppm; HR-MALDI-ToF/MS: *m/z*: calc. for $C_{27}H_{25}NO_5$ $[M+Na]^+$: 466.1630; found 466.1633.



Scheme S2. *Reagents and conditions:* **a**) **5**, TMSOTf (0.3eq), -60ºC, DCM:Et_2O 1:1, 80% ; **b**) THF:Ac_2O:AcOH 3:2:1, Zn-dust, cat. $CuSO_4$ soln., 66% ; **c**) 10% Pd/C, DMF, H_2(g), 60%.

***N*-α-(9-Fluorenylmethyloxycarbonyl)-L-*trans*-4-*O*-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-glucopyranosyl)-proline benzyl ester (7):** A mixture of hydroxy proline acceptor **5** (300 mg, 0.67 mmol), glucosyl trichloroacetaimidate donor **6** (418 mg, 0.88 mmol), and 4Å MS in CH_2Cl_2:diethyl ether, 1:1 (5 ml) was placed under an atmosphere of argon and stirred at room temperature for 1 h. The reaction mixture was then cooled to -60 ^{o}C. TMSOTf (37 µL, 0.20 mmol) was added and stirring was continued for 30 min at the same temperature. The progress of reaction was monitored by TLC and MALDI-ToF MS. The reaction mixture was diluted with CH_2Cl_2 (50 ml), filtered, and washed with sat. aq. $NaHCO_3$ solution (25 ml), water (25 ml), and brine (25 ml). The organic layer was dried ($MgSO_4$), filtered, and the filtrate was concentrated *in*

vacuo. The residue was purified by silica gel column chromatography ($CHCl_3$:Acetone, 98:2, v:v) to afford compound **7** (409 mg, 80%) as white solid. The compound exists as an inseparable mixture of α/β isomers and each isomer in form of a mixture of two rotational isomers. The compound was taken directly for the next step without separation of these isomers.

***N*-α-(9-Fluorenylmethyloxycarbonyl)-L-*trans*-4-*O*-[3,4,6-tri-*O*-acetyl-2-(*N*-acetamido)-2-deoxy-α-D-glucopyranosyl]-proline benzyl ester (8):** Zn dust (420 mg, 6.42 mmol) and saturated aq. $CuSO_4$ (25 µL) were added to a solution of **7** (375 mg, 0.49 mmol) in THF (3 mL), Ac_2O (2 mL), and AcOH (1 mL) and the reaction stirred at rt for 1 h. The reaction mixture was filtered and co-evaporated with toluene (3 x 10 mL). The residue was purified by silica gel column chromatography ($CHCl_3$:Acetone, 9/1, v/v) to afford compound **8** (253 mg, 66%) as pure α-isomer (exists as a mixture of rotational isomers). Analytical data for **8**: R_f = 0.50 ($CHCl_3$:Acetone, 8/2, v/v); ^{1}H-NMR (500 MHz, $CDCl_3/CD_3OD$, 9:1) : δ = 7.72-7.20 (m, 26H, Bn-R_1, Fmoc-R_1, Bn-R_2, Fmoc-R_2), 5.98 (d, 1H, *J* = 8.9 Hz, N*H*Ac-R_1), 5.90 (d, 1H, *J* = 9.2 Hz, N*H*Ac-R_2), 5.17-4.98 (m, 8H, C*H*$_2$Ph-R_1, C*H*$_2$Ph-R_2, H-3R_1, H-4R_1, H-3R_2, H-4R_2), 4.86 (bd, 2H, H-1R_1, H-1R_2), 4.54 (t, 1H, *J* = 7.3 Hz, H-αR_1), 4.48 (bs, 1H, C*H*H-FmocR_1), 4.46 (t, 1H, *J* = 7.0 Hz, H-αR_2), 4.34-4.16 (m, 8H, H-γR_1, C*H*H-FmocR_1, H-γR_2, C*H*$_2$-FmocR_2, H-2R_1, H-2R_2, C*H*-FmocR_1), 4.14 (m, 2H, H-6aR_1, H-6aR_2), 4.03 (bd, 2H, H-6bR_1, H-6bR_2), 3.96 (m, 1H, C*H*-FmocR_2), 3.90 (m, 2H, H-5R_1, H-5R_2), 3.69-3.54 (m, 4H, H-δaR_1, H-δaR_2, H-δbR_1, H-δbR_2), 2.44 (m, 2H, H-βaR_1, H-βaR_2), 2.15 (m, 2H, H-βbR_1, H-βbR_2), 1.96 (s, 12H, 2 x OAcR_1, 2 x OAcR_2), 1.95 (s, 6H, OAcR_1, OAcR_2), 1.80 (s, 6H, NHAcR_1, NHAcR_2) ppm; ^{13}C from HSQC (75 MHz, $CDCl_3$) : δ = 172.07, 171.05, 169.66, 144.19, 143.75, 143.59, 141.48, 130.31, 128.81, 128.58, 128.41, 128.01, 127.32, 125.20, 125.12, 124.00, 120.22, 97.54 (C-1R_1), 96.89 (C-1R_1), 77.47, 76.58, 76.47, 70.99, 70.87, 69.85, 68.56, 68.46, 68.13, 67.45, 62.37, 58.37, 58.00, 52.37, 51.79, 51.64, 47.22, 42.10, 37.44, 36.33, 22.86, 20.83, 20.78, 20.74 ppm; HR-MALDI-ToF/MS: *m/z*: calc. for $C_{41}H_{44}N_2O_{13}$ $[M+Na]^+$: 795.2741; found 795.2738.

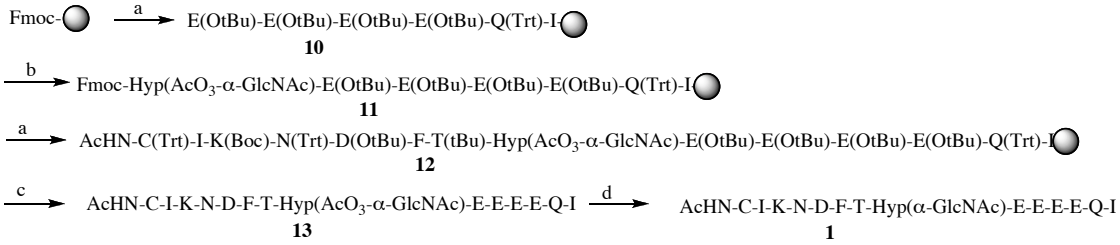
***N*-α-(9-Fluorenylmethyloxycarbonyl)-L-*trans*-4-*O*-[3,4,6-tri-*O*-acetyl-2-(*N*-acetamido)-2-deoxy-α-D-glucopyranosyl]-proline (9):** To a solution of **8** (250 mg, 0.323 mmol) in DMF under an atmosphere of argon was added Pd, 10 wt. % on activated carbon, (125 mg) and the mixture stirred for 20 min. at room temperature. The argon was replaced with H_2, and the reaction stirred for 2 h. The reaction mixture was filtered through celite. The solvent was

removed by evaporation under reduced pressure and the residue purified by silica gel column chromatography ($CHCl_3$:MeOH:AcOH, 99:2:0.5, v:v) to afford compound **9** (135 mg, 61%) as a white solid. Analytical data for **9**: R_f = 0.45 ($CHCl_3$:MeOH:AcOH, 95:5:1, v:v); ^{1}H-NMR (500 MHz, $CDCl_3$/CD_3OD, 9:1) : δ = 7.71-7.24 (m, 8H, Fmoc), 5.86 (m, 1H, N*H*Ac), 5.12 (t, 1H, *J* = 9.6 Hz, H-3), 5.05 (t, 1H, *J* = 9.8 Hz, H-4), 4.87 (bs, 1H, H-1), 4.44 (m, 2H, H-α, C*H*H-Fmoc), 4.36 (bd, 1H, J = 16.0 Hz, H-γ), 4.27 (bd, 1H, *J* = 8.7 Hz, H-2), 4.25-4.13 (m, 3H, C*H*H-Fmoc, C*H*-Fmoc, H-6a), 4.07-4.03 (m, 1H, H-6b), 3.93 (bm, 1H, H-5), 3.67-3.63 (m, 1H, H-δa), 3.53-3.52 (m, 1H, H-δb), 2.44 (m, 1H, H-βa), 2.25 (m, 1H, H-βb), 2.04, 1.97, 1.96 (3 x s, 9H, 3 x OAc), 1.82 (s, 3H, NHAc) ppm; ^{13}C from HSQC (75 MHz, $CDCl_3$) : δ = 170.58, 170.45, 170.09, 169.88, 168.64, 154.07, 143.05, 142.79, 142.64, 140.30, 126.86, 126.16, 124.18, 124.10, 119.07, 96.51 (C-1R_1), 95.70 (C-1R_2), 76.35, 75.51, 69.90, 69.81, 67.54, 67.36, 67.28, 66.96, 61.29, 51.11, 50.60, 50.49, 46.15, 36.33, 21.64, 19.64, 19.62, 19.56 ppm; HR-MALDI-ToF/MS: *m/z*: calc. for $C_{34}H_{38}N_2O_{13}$ $[M+Na]^+$: 705.2272; found 705.2269.

Peptide Synthesis

Peptides were synthesized by established protocols on an ABI 433A peptide synthesizer (Applied Biosystems) equipped with a UV-detector using N^{α}-Fmoc protected amino acids and HBTU plus HOBt as the activating reagents in NMP. The compounds were prepared on a Rink Amide AM resin using the following amino acid building blocks: Fmoc-Ile-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Pro-OH, Fmoc-Hyp(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Phe-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Asn(Trt)-OH, and Fmoc-Lys(Boc)-OH. Single couplings with conditional capping were used for the introduction of all amino acids except for the four N-terminal amino acids Ile, Lys, Asn and Asp for which, it was necessary to use double couplings with capping to achieve satisfactory elongation yields and purity of the crude peptide. To prepare the resin for cleavage it was rinsed with DCM (6 x 5mL) and MeOH (6 x 5 mL) and dried under vacuum. Cleavage of the resin-bound peptide and side-chain deprotection was accomplished by treatment with a mixture of TFA/H_2O/TIS (95% : 2.5% : 2.5%, 20 mL) for 4h. The resin was washed with TFA (2 x 10 mL) and the combined TFA fractions were evaporated to 1/10 of the volume and the crude peptide precipitated with ice-cold *tert*.-butyl methyl ether, washed with *tert*.-butyl methyl ether, and then dissolved in 50% aqueous acetonitrile containing 0.1% TFA and lyophilized.

Reversed phase high performance liquid chromatography (RP-HPLC) was performed on an Agilent 1100 series system equipped with an autosampler, UV-detector and fraction-collector using a Zorbax Eclipse C18 semi-preparative comlumn (5 μm, 25 x 250 mm) at a flow rate of 3 mL/min. All runs used linear gradients of 0-60% solvent B (acetonitrile containing 0.1% TFA) in solvent A (H_2O containing 0.1% TFA) over a 40 min period.



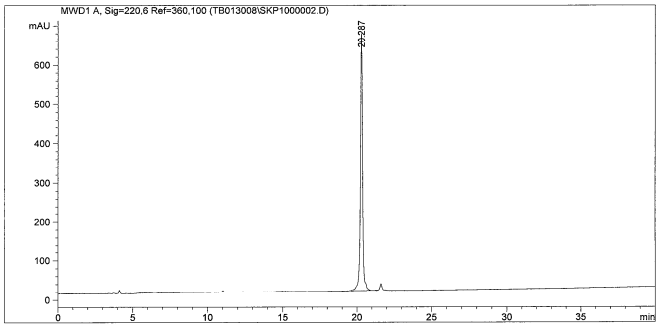
Scheme S3. Synthesis of α-GlcNAc(Hyp) Skp1. *Reagents and Conditions*. **a)** SPPS (Fmoc/tBu) i: 20% piperidine/NMP, ii: protected AA/HBTU/HOBt/DIPEA/NMP; **b)** Fmoc-Hyp(AcO_3-α-D-GlcNAc)-OH, HATU, HOAt, DIPEA, DMF; **c)** Reagent B (94% TFA, 2.5% H_2O, 2.5% EDT, 1% TIS; **d)** 5% aq. hydrazine-hydrate.

Synthesis of Skp1 α-GlcNAcHyp peptide 1.

The glycopeptide was synthesized by SPPS on a Rink Amide AM resin (0.1 mmol) as described above. After automated synthesis of the first 6 amino acids (**10**), coupling of the glycosylated amino acid Fmoc-Hyp-(AcO_3-α-D-GlcNAc)-OH (**9**) (1.5 equiv., 0.15 mmol, 0.102 mg) was carried out manually. HATU (0.15 mmol, 57 mg) and 1-hydroxy-7-azabenzotriazole (HOAt; 0.15 mmol, 20 mmol) were employed as coupling reagents in the presence of DIPEA (0.3 mmol, 52 μL) in DMF. After agitating the resin for 6 h the resin was washed with DMF (4 x 5 mL) and the coupling step was repeated. The manual couplings were monitored by standard Kaiser test. After coupling of the glycosylated amino acid the resin was capped by treatment with 10% acetic anhydride and 5% DIPEA in DMF for 30 min and the resin was then washed thoroughly with DMF (6 x 5 mL). The resin was then returned to the synthesizer and peptide elongation was continued by automated synthesis as described above. Double couplings were employed for all remaining amino acids. The resin (**12**) was rinsed with DCM (6 x 5 mL) and MeOH (6 x 5 mL) and dried under vacuum over night. The resin was placed in a round bottom flask and treated with Reagent B (10 mL, 94% TFA, 2.5% H_2O, 2.5% EDT, 1% TIS) for 1.5 h with occasional swirling. The resin was filtered off, and washed with TFA (2 x 8 mL). The filtrate was evaporated to 1/5 and the peptide was precipitated out using ice-cold diethyl ether. After

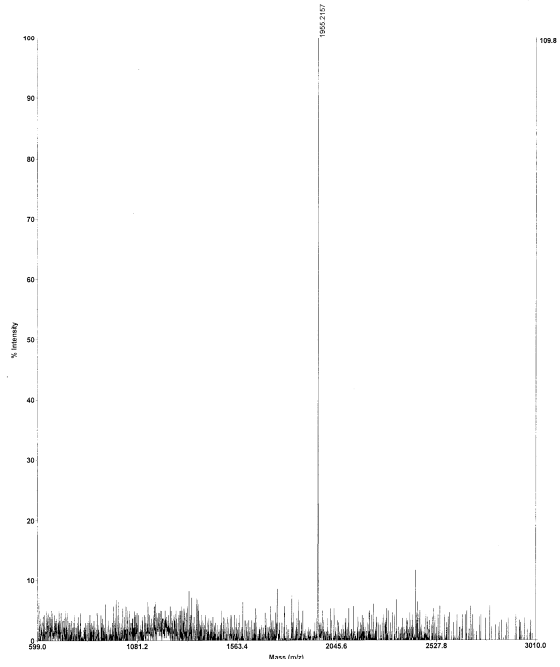
filtration the glycopeptide was dissolved in 50% aqueous acetonitrile containing 0.1% TFA and lyophilized. The crude peptide (**13**) (9.61 μmol, 20 mg) was treated with 5% aqueous hydrazine hydrate (2 mL) and dithiothreitol (DTT; 39 μmol, 6 mg) for 1 h, acetic acid was added, and the mixture was lyophilized. The glycopeptide was purified by semi-preparative C18 reversed-phase HPLC and lyophilized. $C_{82}H_{127}N_{19}O_{34}S$, MALDI-ToF MS: observed $[M+H]^+$, 1955.22 Da; calculated $[M+H]^+$, 1955.06 Da.

HPLC Chromatogram



Column: Semi-prep C18 Reversed phase
Eluent: 0-60% of Solvent B in A over 40 min
Monitor: 215 nm

MALDI-ToF Spectra



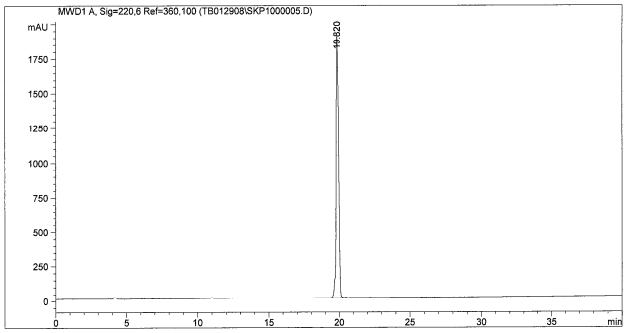
Observed: $[M+H]^+$ 1955.22 Da
Calculated: $[M+H]^+$ 1955.06 Da

Figure S1. HPLC Chromatogram and MALDI ToF spectra of glycopeptide **1**. (Same as Fig. 1B, 1C).

Synthesis of Skp1 Hyp peptide 2.

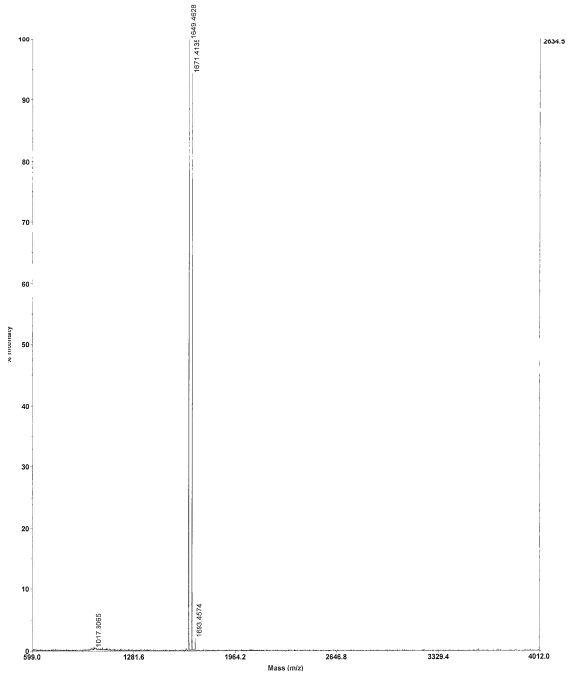
SPPS was performed on Rink Amide AM resin (0.25 mmol) as described above. The crude peptide was purified by semi-preparative C18 reversed-phase HPLC and lyophilized. $C_{71}H_{109}N_{17}O_{28}$, MALDI-ToF MS: observed $[M+H]^+$, 1649.46 Da, $[M^+ + Na^+]$, 1671.41 Da; calculated $[M+H]^+$, 1648.72 Da, $[M+Na]^+$, 1671.71 Da.

HPLC chromatogram



Column: Semi-prep C18 Reversed phase
Eluent: 0-60% of Solvent B in A over 40 min
Monitor: 215 nm

MALDI-ToF Spectra



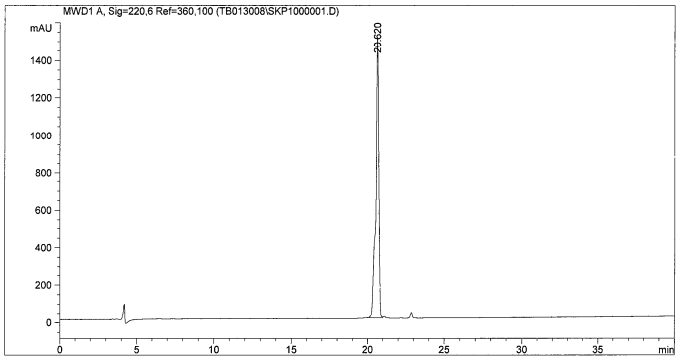
Observed: $[M+H]^+$ 1649.46 Da
$[M+Na]^+$ 1671.41 Da
Calculated: $[M+H]^+$ 1648.72 Da
$[M+Na]^+$ 1671.71 Da

Figure S2. HPLC Chromatogram and MALDI ToF spectra of peptide **2**.

Synthesis of Skp1 Pro peptide 3.

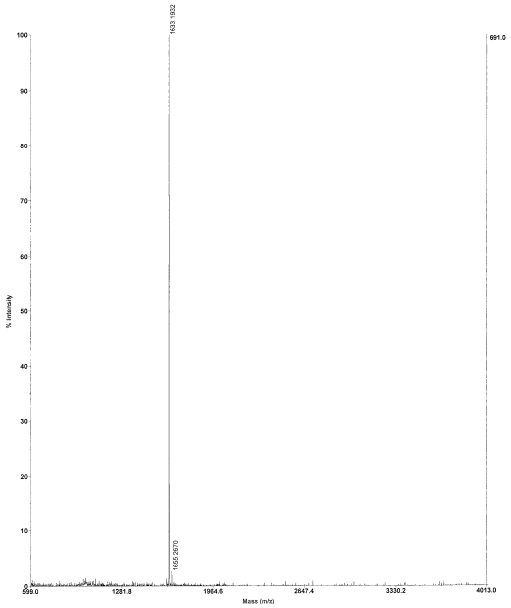
SPPS was performed on Rink Amide AM resin (0.25 mmol) as described above. The crude peptide was purified by semi-preparative C-18 reversed-phase HPLC and lyophilized. $C_{71}H_{109}N_{17}O_{27}$, MALDI-ToF MS: observed $[M+H]^+$, 1633.19; calculated, 1632.72 Da.

HPLC Chromatogram



Column: Semi-prep C18 Reversed phase
Eluent: 0-60% of Solvent B in A over 40 min
Monitor: 215 nm

MALDI-ToF Spectra



Observed: $[M+H]^+$ 1633.19 Da
Calculated: $[M+H]^+$ 1632.72 Da

Figure S3. HPLC Chromatogram and MALDI ToF spectra of peptide **3**.

Conjugation of Glycopeptide 1 to mcKLH: A solution of mcKLH (5.0 mg) in 0.1 M sodium phosphate buffer pH 7.2 containing 0.15 M NaCl (455 μL) was added to a solution of

succinimidyl 3-(bromoacetamido)propionate (SBAP) (2.3 mg) in DMSO (100 μL). The mixture was incubated for 1 h at room temperature and then purified using a D-salt® column (Pierce Endogen) eluated with PBS buffer, 0.9M NaCl, pH 7.2. Fractions containing the activated protein (as determined by Bio-Rad protein assay) were pooled (total volume 1.5 mL) and added to a vial containing glycopeptide **1** (5.0 mg, 2.56 μmol) in PBS buffer pH 7.2 (200 μL). 3M NaOH (6 μL) was added to adjust the pH to 7.5-8.0 (tricolor pH paper). The conjugation mixture was incubated at room temperature for 18 h. Purification was performed as decribed above using a D-Salt® column (Pierce Endogen). This gave a conjugate with 938 residues of **1**/KLH molecule as determined by quantitative monosaccharide analysis by HPAEC/PAD and Bio-Rad protein concentration test.

Conjugation of Glycopeptide 1 to maleimide-activated BSA. Maleimide activated BSA (2.4 mg, lyophilized powder with conjugation buffer sodium phosphate pH 7.2 containing EDTA and sodium azide, Pierce Endogen) was reconstituted with ddH_20 (200 μL), and then added to a vial containing glycopeptide 1 (4.6 mg, 2.36 μmol) in the conjugation buffer (200 μL). The conjugation reaction was stirred for 18 h at ambient temperature after which the conjugate was purified using a D-salt® column (Pierce Endogen) eluated with PBS buffer, 0.9M NaCl, pH 7.2. Fractions containing the activated protein (as determined by Bio-Rad protein assay) were pooled. This gave a conjugate with a glycopeptide **1**/BSA ratio of 12/1 as determined by quantitative monosaccharide analysis by HPAEC/PAD and Bio-Rad protein concentration test.