Supporting Information

Dynamic Glycosylation of the Transcription Factor CREB: A Potential Role in Gene Regulation

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Experimental Section.

General. Unless otherwise noted, reagents were purchased from the commercial suppliers Fisher (Fairlawn, NJ) and Sigma-Aldrich (St. Louis, MO) and were used without further purification. Protease and phosphatase inhibitors were purchased from Sigma-Aldrich or Alexis Biochemicals (San Diego, CA). Bovine GalT and ovalbumin were obtained from Sigma-Aldrich. Uridine diphospho-D-[6-^{3}H]galactose, Redivue L-[^{35}S]methionine (*in vitro* translation grade) and [α-^{32}P] rGTP were purchased from Amersham Biosciences (Piscataway, NJ). Uridine diphosphate *N*-acetyl-D-[6-^{3}H(N)]-glucosamine was purchased from Perkin-Elmer Life Sciences (Boston, MA). Sequencing grade trypsin and chymotorypsin were obtained from Promega (Madison, WI) and Roche Applied Science (Indianapolis, IN), respectively. The MALDI matrix, α-cyano-4-hydroxycinnamic acid, was purchased from Fluka (Milwaukee, WI). Restriction enzymes were obtained from Fisher, with the exception of Nde I (Promega) and Dpn I (New England Biolabs, Beverly, MA).

Preparation of rat brain nuclear extracts. Nuclear extracts were prepared as previously reported[1] with minor modifications. The forebrains of Sprague Dawley rats (Charles River Laboratories, Kingston, MA) were dissected on ice and homogenized in 10 volumes of ice-cold buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) containing protease inhibitors (5 μg/ml pepstatin, 5 μg/ml chymostatin, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 20 μg/ml antipain, 0.2 mM PMSF), phosphatase inhibitors (20 mM NaF, 1 mM Na_3VO_4, 50 μM Na_2MoO_4), and hexosaminidase inhibitors (50 mM GlcNAc and 10 μM streptozocin). The resulting lysate was centrifuged at 1,000xg at 4 ^{o}C for 10 min, and the crude nuclear pellet was washed with buffer A. The pellet was resuspended at 2 mg/ml in buffer B (20 mM HEPES pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 50 mM GlcNAc, 0.2 mM PMSF, 25% (v/v) glycerol) by stirring at 4 ^{o}C for 40 min. Following centrifugation at 10,000xg for 30 min, the supernatant was defined as the nuclear extract. The nuclear extract was dialyzed against buffer D (20 mM HEPES pH 7.9, 0.1 M KCl, 0.5 mM DTT, 0.2 mM EDTA, 50 mM GlcNAc, 20% (v/v) glycerol) for 6 h at 4 ^{o}C, and proteins were precipitated using $(NH_4)_2SO_4$ (38% final concentration). The protein pellet obtained after centrifugation at 21,500xg for 10 min was solubilized in buffer C (0.3 volumes; 25 mM Tris pH7.5, 1 mM EDTA, 1 mM DTT containing protease inhibitors) by gentle mixing for 1 h at 4 ^{o}C. Following clarification at 21,500xg for 5 min, the supernatant was dialyzed into buffer G (20 mM HEPES pH 7.3, 0.1 M KCl, 0.5 mM DTT, 0.2 mM EDTA, 5 mM $MnCl_2$, 0.2 mM PMSF) overnight at 4 ^{o}C. The protein concentration was determined using the BCA assay (Pierce/Endogen Biotech, Rockford, IL).

Labeling with β-1,4-galactosyltransferase (GalT). GalT labeling was performed using minor modifications to published procedures.[2] Nuclear extracts (200-400 μg) were combined with autogalactosylated bovine GalT (100 mU) and 5'-adenosine diphosphate (1.25 mM final concentration). Samples were transferred to centrifuge tubes containing uridine diphospho-D-[6-^{3}H]galactose (0.03 μCi/μl final concentration). Following incubation at 37 °C for 90 min, the labeling reaction was stopped by the addition of 100 mM EDTA (0.1 volumes). Ovalbumin controls were treated in parallel as a positive control.

To label recombinant CREB from Sf9 cells, CREB (2-4 μg) was boiled in 0.1% SDS for 5 min, cooled, and then incubated with GalT (75 mU) and 400 μM UDP-Gal in CREB storage buffer (20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 15% glycerol) plus 5 mM $MnCl_2$, 1.25 mM 5'-adenosine diphosphate and 1% Triton X-100 overnight at 4 °C. Labeling reactions were stopped by the addition of 4x SDS-PAGE loading dye[3] and boiled for 5 min.

Immunoprecipitation of CREB. Radiolabeled nuclear extracts were precleared by incubation with protein A sepharose (Pierce/Endogen Biotech) for 2 h at 4 °C. Samples were centrifuged for 30 s at 2,000xg, and the supernatant was transferred to a fresh centrifuge tube. Samples were then incubated with an antibody selective for CREB (5 μg; Upstate Biotechnology, Lake Placid, NY) or buffer G (negative control) with gentle inversion at 4 °C for 7 h. After 2 h, the samples were supplemented with additional leupeptin (20 μg/ml) and antipain (20 μg/ml) protease inhibitors. Following the incubation period, protein A sepharose was added, and the samples were incubated for 2 h at 4 °C. Samples were then centrifuged for 30 s at 2,000xg, and the sepharose was washed twice with buffer G and four times with PBS (137 mM NaCl, 2.7 mM KCl, 5 mM Na_2HPO_4, 1.7 mM KH_2PO_4). Proteins were eluted with boiling in 1% SDS, 1% β-mercaptoethanol (BME) for 10 min.

PNGase F treatment. An equal volume of 2xPNGase buffer (150 mM Na_2HPO_4 pH 8.6, 15 mM EDTA, 5% NP-40) containing protease inhibitors was added to the eluted protein or to the ovalbumin control (boiled in 1% SDS, 1% BME). Samples were incubated in the presence or absence of PNGase F (2 U; New England Biolabs) for 5 h with rocking at 25 °C. Additional PNGase F (1 U) was added, and the incubation continued for 5 h.[2] Samples were then boiled for 5 min in SDS-PAGE loading dye and resolved by SDS-PAGE.[3] Gels were stained in 0.1% Coomassie Brilliant Blue, 50% MeOH and 5% AcOH, for 15 min and destained in 50% MeOH, 5% AcOH. Gels were treated with Amplify solution (Amersham Biosciences) for 30 min, rinsed with 2% glycerol and dried. Tritium-labeling was visualized using Hyperfilm™ MP (Amersham Biosciences) exposed at -80 °C.

Purification of CREB and OGT from *Spodoptera frugiperda* (Sf9) cells. Rat CREB and human OGT cDNA clones were generously provided by Dr. R. H. Goodman (Oregon Health & Science University)[4] and Dr. J. A. Hanover (NIDDK, National Institutes of Health),[5] respectively, and were cloned into baculovirus expression vectors in frame with a histidine tag. Baculovirus preparation and protein expression in Sf9 cells were performed by Dr. P. Snow at the Beckman Institute Protein Expression Facility at the California Institute of Technology.[6] Proteins were purified using Ni-NTA agarose (Qiagen, Valencia, CA) with the following modifications to the manufacturer's protocol. For optimal solubility, the lysis, wash and elution buffers were supplemented with 10 mM BME and 0.1% Triton X-100. Purified CREB and OGT were dialyzed into CREB storage buffer or OGT storage buffer (50 mM Tris-HCl pH 7.5, 12.5 mM $MgCl_2$, 40% glycerol) and stored at -80 °C.

The relative levels of *O*-GlcNAc glycosylation were compared for CREB expressed in the absence and presence of OGT as follows. After labeling with GalT, CREB was resolved by SDS-PAGE. The gels were stained with Coomassie and treated with Amplify solution as described above. The relative glycosylation levels were measured by analysis of fluorography and Coomassie images using NIH Image 1.52 software. The data, which were normalized for CREB concentration, showed a 5.8-fold increase in the level of CREB glycosylation upon co-expression with OGT. As described below, similar values were obtained by LC-MS/MS analysis.

Purification of CREB from rat brain. CREB was purified from rat brain nuclear extracts by DNA affinity chromatography using minor modifications to published procedures.[12] The cAMP-Response Element (CRE) DNA affinity column was prepared as follows. Single-stranded oligonucleotides containing the CRE sequence and BamHI restriction sites (5'-GGA TCC GCC **TGA CGT CA**G AG-3' and 5'-GGA TCC CTC **TGA CGT CA**G GC-3'; the CRE sequence is shown in boldface) were annealed and 5' phosphorylated with polynucleotide kinase (Roche Applied Sciences) in 50 mM Tris-HCl pH 8.2, 10 mM $MgCl_2$, 10 μM EDTA, 5 mM DTT, 0.1 mM spermidine, 3 mM ATP for 3 h at 37 ^{o}C. The phosphorylated oligonucleotides (~40 μM) were then ligated with T4 DNA ligase (Roche Applied Sciences) in 66 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 4 mM ATP overnight at 16 ^{o}C. Following the ligation, the CRE oligomers were precipitated with ethanol and resolubilized in the coupling buffer (0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl; 2 mg/mL). Coupling of the oligomers to CNBr-activated sepharose (Amersham Biosciences) was performed according to the manufacturer's protocol, using 4 mg of CRE oligomers per mL of sepharose.

Nuclear extracts were prepared from 30 rat forebrains as described above and dialyzed into buffer E (50 mM Tris-HCl pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 20% (v/v) glycerol, 0.1% (v/v) NP-40). Extracts (2 mg/mL) were incubated with an equal volume of CRE affinity sepharose for 10 h at 4 °C. The sepharose was washed with 5 volumes of buffer E, and proteins bound to the column were eluted with 2.5 volumes of buffer E containing 1 M KCl. Fractions enriched in CREB were identified by Western blot analysis, precipitated with 10% trichloroacetic acid/acetone, and resolved by SDS-PAGE. The CREB protein was excised from the Coomassie-stained gels, digested with trypsin, and analyzed by MALDI-TOF MS and LC-MS/MS.

Tryptic and chymotryptic in-gel digests and MALDI-MS analysis. Tryptic digests were performed using methods developed by Dr. G. Hathaway at the Beckman Institute Protein/Peptide Microanalysis.[7] Digests with chymotrypsin were performed according to manufacturer's protocol. Samples were concentrated on C18 zip tips (Millipore, Bedford, MA) and were combined with the MALDI matrix (1 volume of saturated α-cyano-4-hydroxycinnamic acid in 50% aqueous CH_3CN with 0.1% TFA). All MALDI spectra were acquired on a PerSeptive Biosystems Voyager-DE Pro at 20,000 kV in the reflector mode. Samples were internally calibrated based on the monoisotopic *m/z* of tryptic peptides calculated using Protein Prospector v 4.0.4.[8] CREB coexpressed in Sf9 cells with OGT exhibited identical fragmentation patterns to CREB expressed alone, although as anticipated, the intensities of the glycopeptide ions were lower in the absence of OGT.

LC-MS/MS analysis of recombinant and native CREB. Liquid chromatography and tandem mass spectrometry (LC-MS/MS) were performed on an LCQ Classic ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) that was fitted with a laboratory-built nanospray source and interfaced with a Surveyor HPLC system (ThermoFinnigan, San Jose, CA). Tryptic digests of

recombinant CREB were analyzed using a PicoFrit™ capillary column (0.075 mm i.d. x 50 mm; New Objective, Woburn, MA) packed with 5µ 100Å C18 reverse phase (RP) particles (Magic C18, Michrom BioResources, Auburn, CA). All other digests were analyzed using a Michrom BioResources capillary column (0.3mm i.d. x 150mm) pre-packed with 5µ 200Å C18 RP particles. To achieve microflow rates compatible with capillary chromatography (~1µL/min), the flow from the HPLC was reduced with a splitting tee and a 150 mm section of fused silica tubing (0.05 i.d.). Approximately 2 pmoles of the tryptic digests were loaded on the microcolumns using the Surveyor Autosampler (ThermoFinnigan) and separated by RP chromatography. The LC buffers used were A: 2% CH_3CN, 0.1% AcOH, 0.005% heptafluorobutyric acid (HFBA), 97.9% H_2O and B: 90% CH_3CN, 0.1% AcOH, 0.005% HFBA, 9.9% H_2O. The gradient, which was optimized for separation of the glycosylated peptides, consisted of: 0-5 min, 0% B; 5-10 min, 0-18% B; 10-50 min, 18-40% B; 50-55 min, 40-100% B; 55-60 min, 100%.

The LCQ was operated in automated mode using the Xcalibur™ software of the LCQ. The nanospray voltage was 1.8 kV and the heated capillary was 170-180 ˚C. Automatic gain control was active, and the ion injection time was set at 200 ms for full scan mode of operation (3 scans per scan) or 400 ms (5 scans per scan) for MS/MS mode. Dynamic exclusion was used during data acquisition to ensure that the majority of co-eluting peaks would be selected for collision-induced dissociation (CID). In this mode of analysis, the ion trap acquires full scan MS spectra until an ion is present in a scan above a specified threshold. This triggers the ion trap to isolate that ion and generate a product ion spectrum (MS/MS). The ion trap returns to full scan operation when the ion intensity drops below the specified threshold. For native CREB, a parent ion of 1512.8 *m/z*, corresponding to $[M+GlcNAc]^{2+}$ was selected for MS/MS analysis. Relative collision energy for CID was preset to 35% and a default charge state of +2 was selected to calculate the scan range for acquiring tandem MS spectra. The precursor ion isolation window was set at 2.5 for maximum sensitivity.

Supplementary Figure 1 shows selective ion chromatograms for four distinct forms of a chymotryptic peptide corresponding to residues 253-268 of CREB. As expected, sequential elution of the glycoforms was observed by reverse-phase LC, with the diglycosylated form (A) eluting first, followed by the monoglycosylated forms (B and B'), and then the unglycosylated peptide (C). Representative MS/MS spectra of the glycoforms B and B' ($[M+GlcNAc]^{2+}$ = 922.9 *m/z*) are shown in Supplementary Figure 2. The major ion observed after CID is the parent ion minus GlcNAc ($[M+2H]^{2+}$ = 821 *m/z*). Supplementary Table 1 shows all of the peptide fragments observed by LC-MS/MS for each of the four forms.

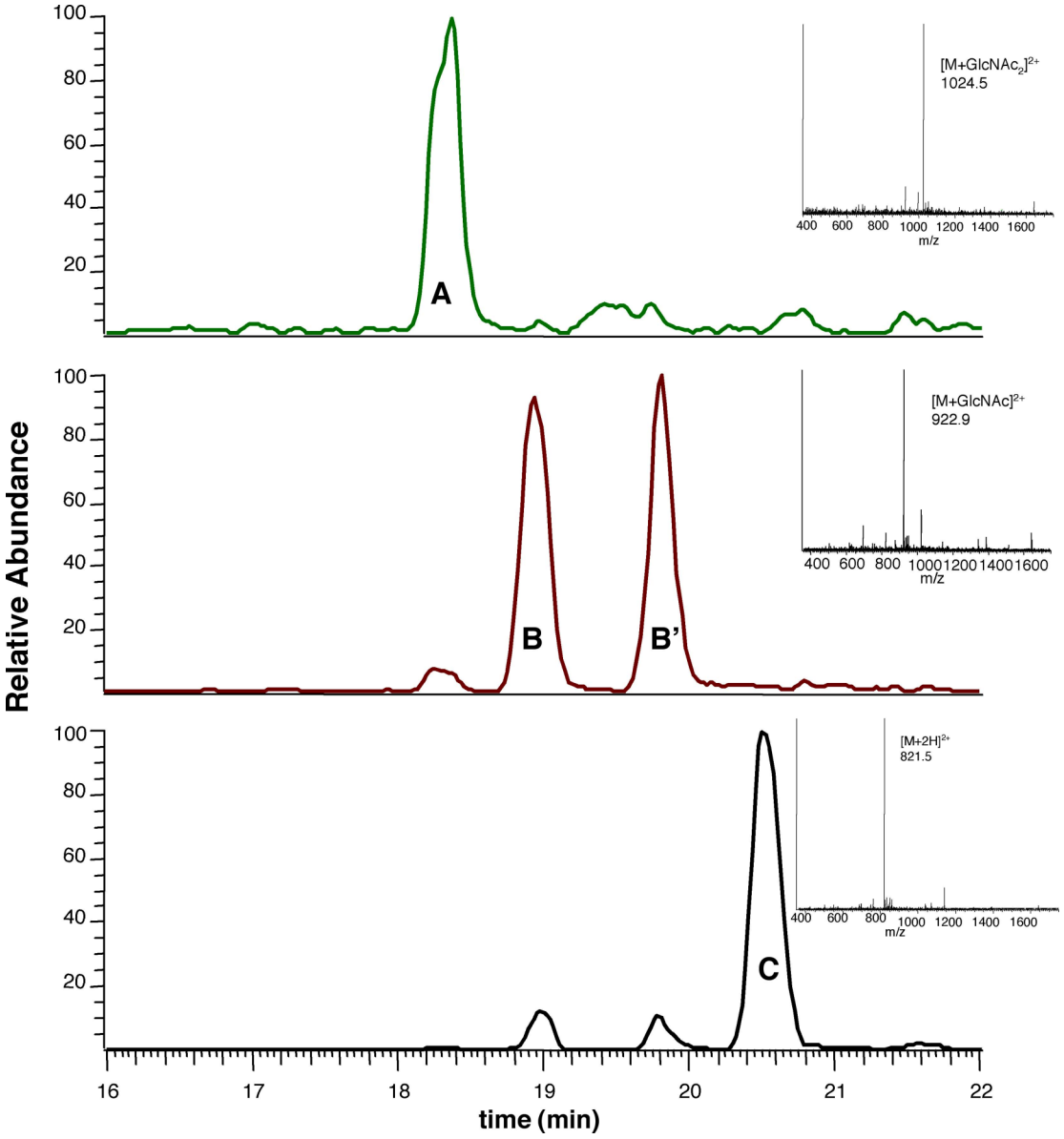
Supplementary Figures 3 and 4 show selective ion chromatograms and representative MS/MS spectra for the monoglycosylated peptides obtained from tryptic digestion of recombinant or native CREB, respectively. For native CREB, LC-MS experiments were run in single ion monitoring (SIM) mode with selected parent ions: 1512.8 *m/z* ($[M+GlcNAc]^{2+}$, where M = ^{256}TAPTSTIAPGVVMASSPALPTQPAEEAAR^{284}) and 1411.4 *m/z* ($[M+2H]^{2+}$). The presence of two $[M+GlcNAc]^{2+}$ species (1512.8 *m/z*) that produce $[M+2H]^{2+}$ fragments (1411.4 *m/z*) confirmed their assignment as monoglycosylated forms of the CREB peptide (Supplementary Figure 4a). The MS/MS spectra of the monoglycosylated forms of recombinant and native CREB are similar (Supplementary Figures 3b and 4b). The native CREB showed fragment ions of weaker relative abundance due to the smaller amount of protein available for analysis.

To calculate the stoichiometry of glycosylation within region 256-284, the on-line LC-MS peaks assigned to the four distinct forms of peptide 256-284 were integrated using the Xcalibur software. CREB expressed in the presence and absence of OGT exhibited 55.4% and 9.6%

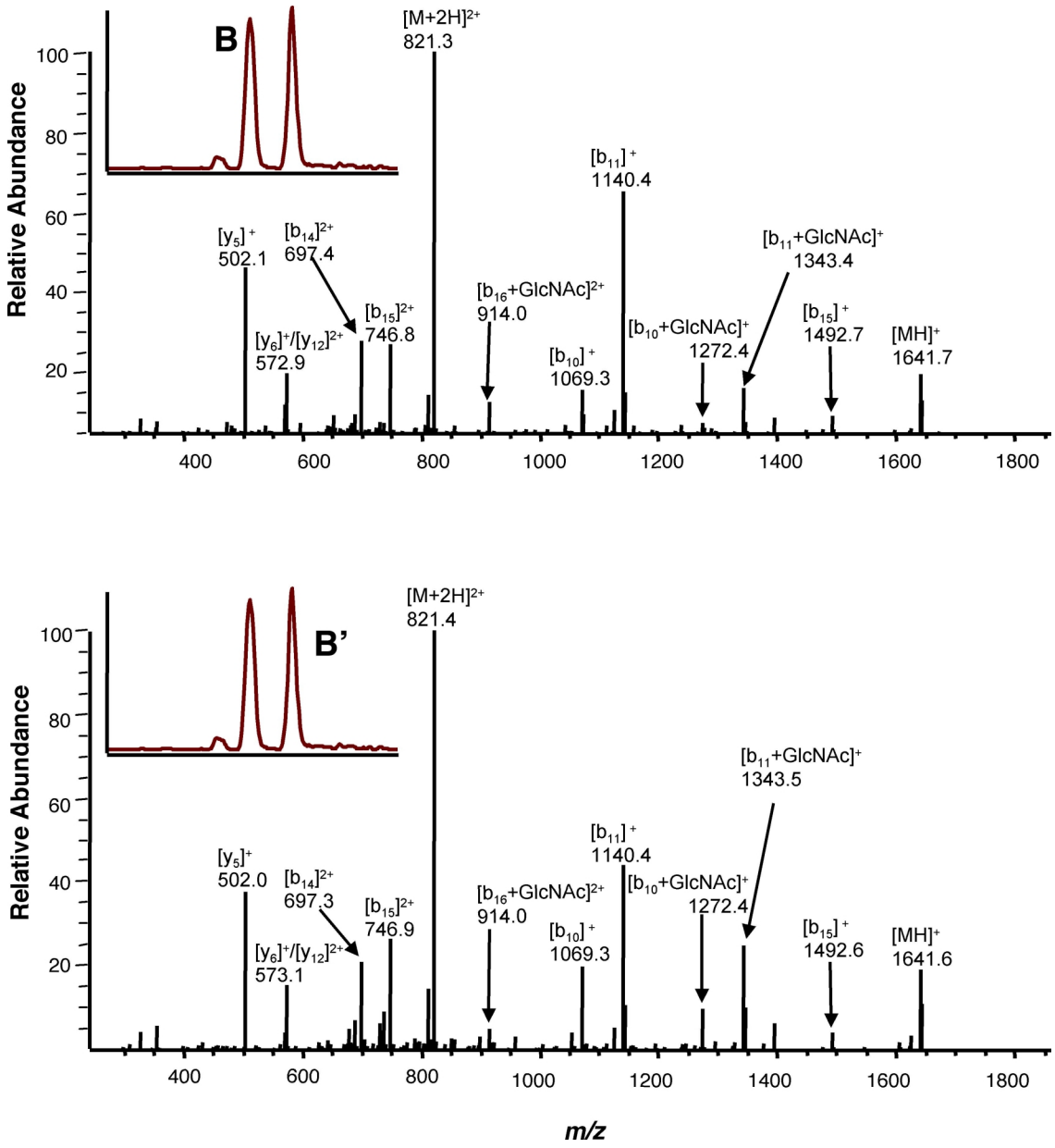
glycosylation, respectively. Native CREB purified from rat brain contained approximately 0.6% glycosylation.

$TAF_{II}130$ binding assay. [^{35}S]-labeled $TAF_{II}130$ was synthesized using the TNT T7 Coupled Reticulocyte Lysate System (Promega) following the manufacturer's protocol. The $TAF_{II}130$ construct, pTβ-h$TAF_{II}130$, was kindly provided by Dr. N. Tanese (New York University School of Medicine).[9] Binding assays were performed with minor modifications to published procedures.[10,11] Proteins (50-100 μg CREB purified from Sf9 cells; 100-200 μg OGT purified from Sf9 cells as a control) were incubated with Ni-NTA agarose for 4-5 h at 4 ^{o}C with mixing. Following centrifugation, the agarose was washed twice with buffer H (20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 0.5 mM DTT, 0.1% NP-40, 20% glycerol, 0.1 mg/ml BSA)[9] containing 50 mM imidazole and twice with buffer H. The agarose was then incubated with [^{35}S]-labeled $TAF_{II}130$ lysate (0.13 volumes) in buffer H (2.5 volumes) for 4 h at 4 ^{o}C with mixing.[10,11] Following centrifugation, the agarose was washed five times with buffer H containing 50 mM imidazole. Samples were boiled in SDS-PAGE loading dye and resolved by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue, incubated with Amplify, and dried. The relative binding affinity of $TAF_{II}130$ was visualized by autoradiography on Hyperfilm and quantified by analysis of scanned images using NIH Image 1.52 software. Values were normalized for the concentration of CREB in each experiment, as determined by Coomassie staining and densitometry. Data were analyzed by the Student's unpaired t-test using StatView 5.0.1 software, with significance defined as $p < 0.05$. Supplementary Figure 5 shows representative autoradiograms and Coomassie stained gels.

***In vitro* transcription assay.** Assays were performed using HeLaScribe Nuclear Extract (Promega) according to the manufacturer's protocol with minor modifications. Linearized CRE template DNA was prepared by digesting the plasmid pCRE-Luc (Stratagene, La Jolla, CA) with Nde I and EcoRV. The resulting 2.1kb fragment containing the CRE enhancer and partial luciferase ORF (~1.4kb) was gel purified. CREB (0.3 - 0.5 μg) purified from Sf9 cells with or without OGT was combined with the CRE template DNA (100 ng), 0.4 mM rATP, 0.4 mM rUTP, 0.4 mM rCTP, 0.016 mM rGTP, and 10 μCi [α-^{32}P]rGTP (3000 Ci/mmol, Amersham Biosciences) in 7.3 mM HEPES pH 7.9, 6.1 mM $MgCl_2$, 37 mM KCl, 0.07 mM EDTA, 0.2 mM DTT, 5.5% glycerol. Transcription reactions were initiated by the addition of HeLaScribe nuclear extract (8 U). Run-off RNA transcripts were resolved on 7 M urea 6% TBE-PAGE gels, visualized by autoradiography using Hyperfilm MP film, and quantified by analysis of scanned images using NIH Image 1.52 software. Values for each reaction were corrected for CREB and [α-^{32}P] rGTP concentration, as determined by Coomassie staining and autoradiography, respectively. Data were analyzed by the Student's unpaired t-test using StatView 5.0.1 software, with significance defined as $p < 0.05$.



Supplementary Figure 1. Reverse phase LC-MS analysis of CREB coexpressed with OGT. Selective ion chromatograms reveal four distinct forms of a CREB peptide. Diglycosylated peptide (A) eluted first from the C18 column, followed by two monoglycosylated peptides (B and B') and the unglycosylated peptide (C). M = ^{253}QIRTAPTSTIAPGVVM^{268}.



Supplementary Figure 2. LC-MS/MS analysis of the chymotryptic CREB ions of 922.9 *m/z* , B and B'. CID sequence analysis positively identified both peaks as monoglycosylated forms of the CREB peptide, M= ^{253}QIRTAPTSTIAPGVVM^{268}. The most intense ion in the CID spectrum (821 *m/z*) represents the parent ion minus GlcNAc.

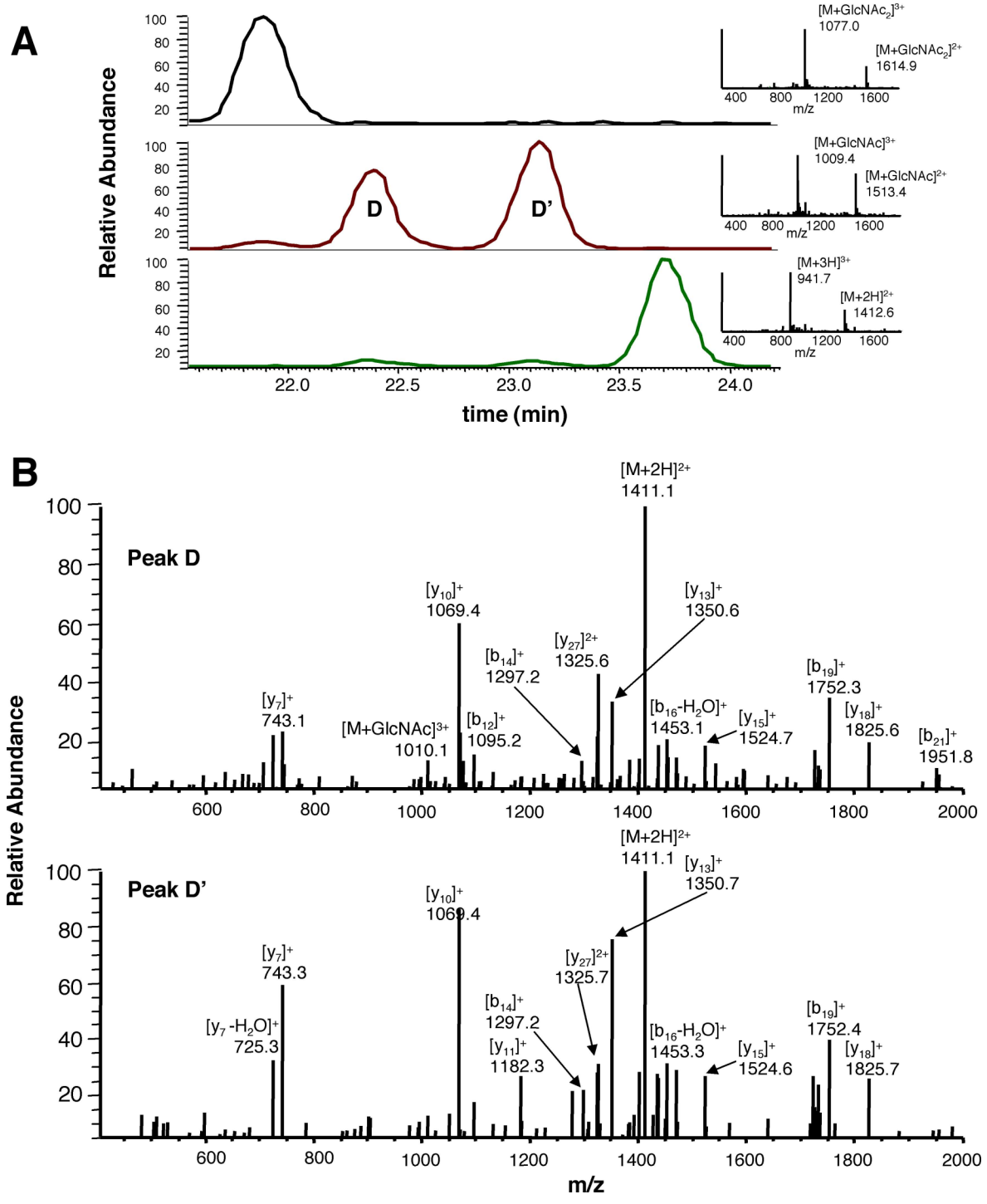
Supplementary Table 1. Summary of all fragment ions observed by LC-MS and tandem MS for CREB peptide ^{253}QIRTAPTSTIAPGVVM^{268} generated by digest with chymotrypsin.

	observed m/z	calculated m/z	assignment	amino acids
A : Di-glycosylated peptide	1069.3	1069.6	$[b_{10}]^+$	QIRTAPTSTI
	1642.5	1641.9	$[M+H]^+$	QIRTAPTSTIAPGVVM
	923.7	922.9	$[M+GlcNAc]^{2+}$	QIRTAPTSTIAPGVVM + GlcNAc
	1024.4	1024.5	$[M+GlcNAc_2]^{2+}$	QIRTAPTSTIAPGVVM + $GlcNAc_2$

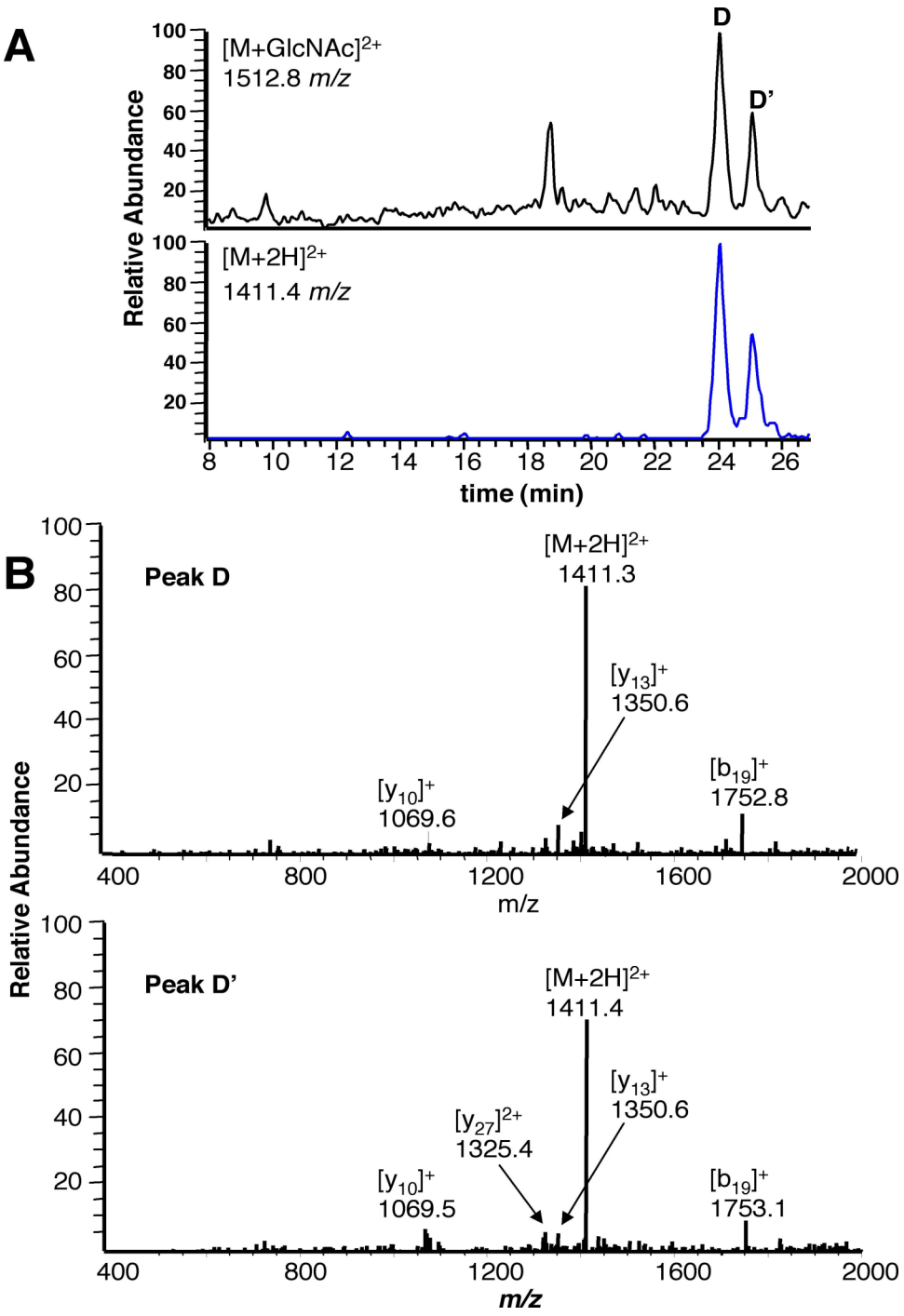
	observed m/z	calculated m/z	assignment	amino acids
B : Mono-glycosylated peptide	1069.3	1069.6	$[b_{10}]^+$	QIRTAPTSTI
	1272.4	1272.7	$[b_{10}+GlcNAc]^+$	QIRTAPTSTI + GlcNAc
	1140.4	1140.6	$[b_{11}]^+$	QIRTAPTSTIA
	1343.4	1343.7	$[b_{11}+GlcNAc]^+$	QIRTAPTSTIA + GlcNAc
	1393.6	1393.8	$[b_{14}]^+$	QIRTAPTSTIAPGV
	697.4	697.8	$[b_{14}]^{2+}$	QIRTAPTSTIAPGV
	1492.7	1492.8	$[b_{15}]^+$	QIRTAPTSTIAPGVV
	746.8	747.4	$[b_{15}]^{2+}$	QIRTAPTSTIAPGVV
	914.0	914.5	$[b_{16}+GlcNAc]^{2+}$	QIRTAPTSTIAPGVVM + GlcNAc
	502.1	502.3	$[y_5]^+$	PGVVM
	572.9	573.3	$[y_6]^+$ or $[y_{12}]^{2+}$	APGVVM
	1641.7	1641.9	$[M+H]^+$	QIRTAPTSTIAPGVVM
	821.3	821.5	$[M+2H]^{2+}$	QIRTAPTSTIAPGVVM
	923.0	923.0	$[M+GlcNAc]^{2+}$	QIRTAPTSTIAPGVVM + GlcNAc

	observed m/z	calculated m/z	assignment	amino acids
B' : Mono-glycosylated peptide	1069.3	1069.6	$[b_{10}]^+$	QIRTAPTSTI
	1272.4	1272.7	$[b_{10}+GlcNAc]^+$	QIRTAPTSTI + GlcNAc
	1140.4	1140.6	$[b_{11}]^+$	QIRTAPTSTIA
	1343.5	1343.7	$[b_{11}+GlcNAc]^+$	QIRTAPTSTIA + GlcNAc
	1393.5	1393.8	$[b_{14}]^+$	QIRTAPTSTIAPGV
	697.3	697.8	$[b_{14}]^{2+}$	QIRTAPTSTIAPGV
	1492.6	1492.8	$[b_{15}]^+$	QIRTAPTSTIAPGVV
	746.9	747.4	$[b_{15}]^{2+}$	QIRTAPTSTIAPGVV
	914.1	914.5	$[b_{16}+GlcNAc]^{2+}$	QIRTAPTSTIAPGVVM + GlcNAc
	502.0	502.3	$[y_5]^+$	PGVVM
	573.1	573.3	$[y_6]^+$ or $[y_{12}]^{2+}$	APGVVM
	1641.6	1641.9	$[M+H]^+$	QIRTAPTSTIAPGVVM
	821.4	821.5	$[M+2H]^{2+}$	QIRTAPTSTIAPGVVM
	922.9	923.0	$[M+GlcNAc]^{2+}$	QIRTAPTSTIAPGVVM + GlcNAc

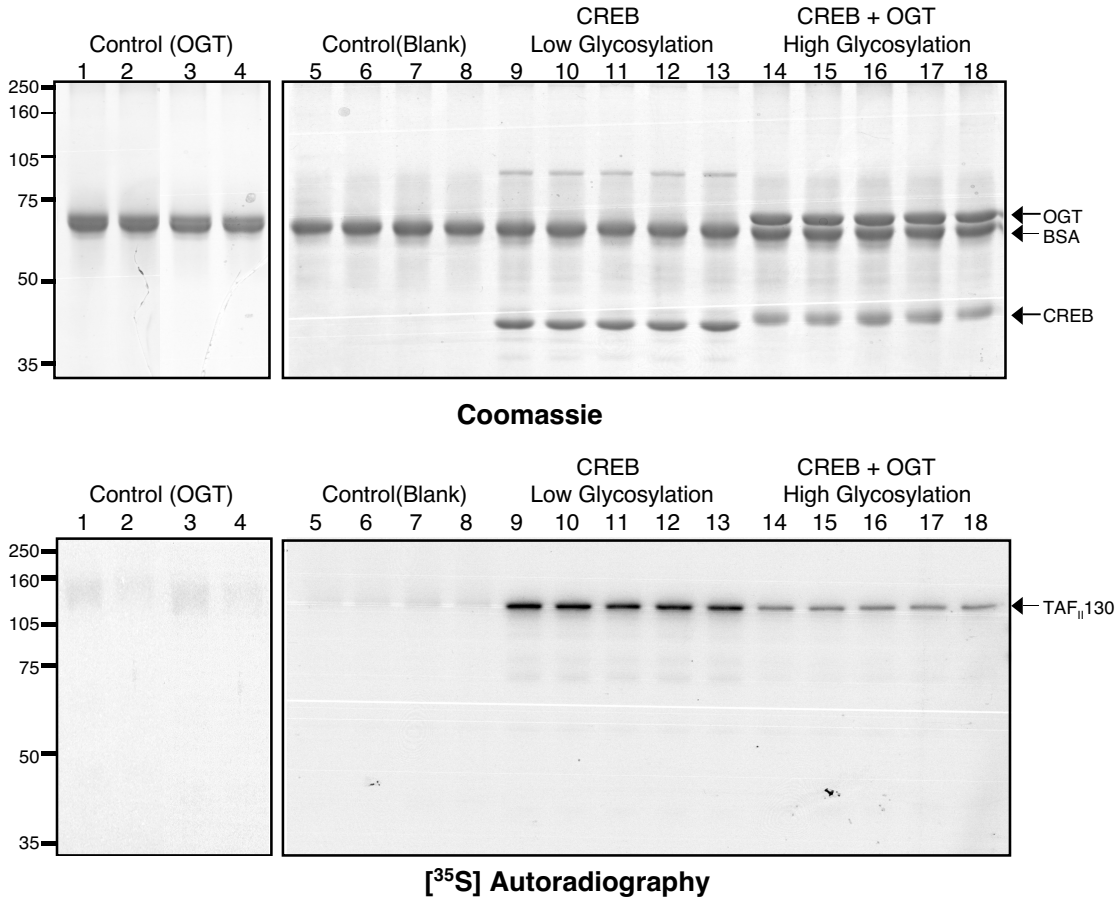
	observed m/z	calculated m/z	assignment	amino acids
C : Unglycosylated peptide	1069.4	1069.6	$[b_{10}]^+$	QIRTAPTSTI
	1140.4	1140.6	$[b_{11}]^+$	QIRTAPTSTIA
	1393.6	1393.8	$[b_{14}]^+$	QIRTAPTSTIAPGV
	697.2	697.8	$[b_{14}]^{2+}$	QIRTAPTSTIAPGV
	746.9	747.4	$[b_{15}]^{2+}$	QIRTAPTSTIAPGVV
	812.4	813	$[b_{16}]^{2+}$	QIRTAPTSTIAPGVVM
	502.1	502.3	$[y_5]^+$	PGVVM
	573.0	573.3	$[y_6]^+$ or $[y_{12}]^{2+}$	APGVVM
	1641.6	1641.9	$[M + H]^+$	QIRTAPTSTIAPGVVM
	821.5	821.5	$[M +2H]^{2+}$	QIRTAPTSTIAPGVVM



Supplementary Figure 3. LC-MS/MS analysis of recombinant CREB coexpressed with OGT. (A) Selective ion chromatograms reveal two distinct monoglycosylated forms of a tryptic CREB peptide (D and D'; $[M+GlcNAc]^{2+} = 1513$ *m/z*). (B) Representative tandem mass spectra of the monoglycosylated peptides D and D'. The most intense ion in the CID spectrum (1411.1 *m/z*) represents the parent ion minus GlcNAc. M = ^{256}TAPTSTIAPGVVMASSPALPTQPAEEAAR^{284}.



Supplementary Figure 4. LC-MS/MS analysis of native CREB purified from rat brain. (A) Selective ion chromatograms reveal two distinct monoglycosylated forms of a tryptic CREB peptide (D and D'; $[M+GlcNAc]^{2+}$ = 1513 *m/z*). The 1411.4 *m/z* selective ion chromatogram shows that only peaks D and D' generate daughter ions equivalent to the loss of GlcNAc. The minor difference in retention times of D and D' between recombinant and native CREB is due to the use of different HPLC columns. (B) Representative tandem mass spectra of the monoglycosylated peptides D and D'. M = ^{256}TAPTSTIAPGVVMASSPALPTQPA EEAAR^{284}.



Supplementary Figure 5. Representative Coomassie-stained gel (*upper panel*) and corresponding autoradiogram (*lower panel*) from the $TAF_{II}130$ binding assay. Lanes 1-4: controls with OGT alone; Lanes 5-8: controls in the absence of CREB; Lanes 9-13: CREB purified from Sf9 cells (low glycosylation stoichiometry); lanes 14-18: CREB co-expressed with OGT and purified from Sf9 cells (high glycosylation stoichiometry). Four or five duplicate runs are shown for each sample. Note that CREB co-expressed with OGT has a higher apparent molecular weight than CREB alone. BSA is present in all lanes due to its presence in incubation and wash buffers.

References.

1. Andrews, N. C.; Faller, D. V. *Nuc. Acid Res*. **1991**, *19*, 2499.
2. Roquemore, E. P.; Chow, T. Y.; Hart, G. W. *Methods Enzymol*. **1994**, *230*, 443-461.
3. Sambrook, J.; Fritsch, E. F.; Maniatis, T. In *Molecular Cloning: A Laboratory Manual*; Ford, N.; Nolan, C.; Ferguson, M., Eds.; Cold Spring Harbor Laboratory Press: New York, 1989; Book 3, pp 18.53.
4. Loriaux, M. M.; Rehfuss, R. P.; Brennan, R. G.; Goodman, R. H. *Proc. Natl. Acad. Sci. U.S.A*. **1993**, *90*, 9046-9050.
5. Lubas, W. A.; Frank, D. W.; Krause, M.; Hanover, J. A. *J. Biol. Chem*. **1997**, *272*, 9316-9324.
6. Strong, R. K.; Vaughn, D. E.; Bjorkamn, P. J.; Snow. P. M. *J. Mol. Biol*. **1994**, *241*, 483-487.
7. Zhou, J.; Rusnak, F.; Colonius, T.; Hathaway, G. M. *Rapid Commun. Mass. Spectrom*. **2000**, *14*, 432-438.
8. Clauser K. R.; Baker P. R.; Burlingame A. L. *Anal. Chem*., **1999**, *71*, 2871-2872.
9. Tanese, N.; Saluja, D.; Vassallo, M. F.; Chen, J-L.; Admon, A. *Proc. Natl. Acad. Sci. U.S.A*. **1996**, *93*, 13611-13616.
10. Parker, D.; Ferreri, K.; Nakajima, T.; LaMorte, V. J.; Evans, R.; Koerber, S. C.; Hoeger, C.; Montminy, M. R. *Molec. Cell. Biol*. **1996**, *16*, 694-703.
11. Kim, L. J.; Seto, A. G.; Nguyen, T. N.; Goodrich, J. A. *Molec. Cell. Biol*. **2001**, *21*, 3503-3513.
12. Kadonga, J. T.; Tjian, R. *Proc. Natl. Acad. Sci. U.S.A*. **1986**, *83*, 5889-5892.