Supporting Information

for

"Comprehensive Characterization of ^{S}GTP-binding Proteins by Orthogonal Quantitative ^{S}GTP-affinity Profiling and ^{S}GTP/GTP Competition Assays"

by

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Anal. Chem., 2014

Supplementary Experimental Details

Preparation of the Biotinylated Nucleotide Affinity Probe

The biotinylated nucleotide affinity probes were prepared according to previously published procedures with minor modifications[1]. In this regard, we first prepared desthiobiotinyl-aminobutyric acid (desthiobiotin-C4) following previously published procedures. Isobutyl chloroformate (0.19 mL) was added to a solution containing 150 mg desthiobiotin, 15 mL DMF, and 0.38 mL tri-*n*-butylamine. After being incubated at room temperature for 10 min, the mixture was slowly added to a suspension of 230 mg γ-aminobutyric acid (Sigma) in DMF (7.5 mL) at 5°C. After stirring at 5°C for 2 h, the solvent was removed under reduced pressure and the crude product was dissolved in 5 mL water at 40°C. The solution pH was adjusted to 2 with 2.0 N HCl, and the mixture was kept at 0°C for 12 h. The desired desthiobiotin-C4 was precipitated out of solution. The precipitate was filtered, washed with water, and dried under vacuum.

To render nucleotide soluble in organic solvent, the commercially available sodium salt form of GTP or synthesized ^{S}GTP was first converted to the tributylammonium form by passing the nucleotides through a cation-exchange column packed with Spectra/Gel IE 50×8 resin (40-75 μm) at 4°C once. Fractions containing the tributylammonium form of GTP or ^{S}GTP were collected and lyophilized. Desthiobiotin-C4 (8 mg), dissolved in a 1-mL solvent mixture of ice-cold dry CH_2Cl_2 and DMF (4:1, v/v), was mixed with tri-*n*-butylamine (11 μL) and ethyl chloroformate (5 μL). After stirring at 0°C for 5 min, the mixture was stirred at room temperature under argon atmosphere for another 60 min. Tributylammonium form of GTP or ^{S}GTP (50 mg), dissolved in a 1.25 mL solution of CH_2Cl_2 and DMF (4:1, v/v), was then added to the above reaction mixture. The reaction was continued at room temperature and under argon atmosphere for 18 h. The CH_2Cl_2 was then removed by purging with argon for 10 min and the remaining 200 μL solution was directly subjected to HPLC purification with a YMC ODS-AQ column (4.8×250 mm, 120 Å in pore size, 5 μm in particle size, Waters). The flow rate was 0.8 mL/min, and a 45 min linear gradient of 0-30% acetonitrile in 50 mM triethylammonium acetate (pH 6.8) was used for the purification. A UV detector was set at 253 and 340 nm to monitor the effluents for GTP and ^{S}GTP probes, respectively. Appropriate HPLC fractions were pooled, lyophilized, and stored at -80°C. The structures of the products were confirmed by ESI-MS (Supplementary Figure 1B)[1].

LC-MS/MS Analysis

LC-MS/MS analysis was performed on an LTQ-Orbitrap Velos mass spectrometer equipped with a nanoelectrospray ionization source (Thermo Fisher Scientific, San Jose, CA). Samples were automatically loaded from a 48-well microplate autosampler using an EASY-nLC system (Proxeon Biosystems, Odense, Denmark) at 3 μL/min onto a home-made 4 cm trapping column (150 μm i.d.) packed with 5 μm C18 120 Å reversed-phase material (ReproSil-Pur 120 C18-AQ, Dr. Maisch). The trapping column was connected to a 20 cm fused silica analytical column (PicoTip Emitter, New Objective, 75 μm i.d.) with 3 μm C18 beads (ReproSil-Pur 120 C18-AQ, Dr. Maisch). The peptides were then separated with a 120-min linear gradient of 2-35% acetonitrile in 0.1% formic acid and at a flow rate of 250 nL/min. The LTQ-Orbitrap Velos was operated in a data-dependent scan mode. Full-scan mass spectra were acquired in the Orbitrap analyzer with a resolution of 60000 with lock mass option enabled for the ion of *m/z* 445.120025 [2]. Up to 20 most abundant ions found in MS with charge state $\geq$2 were sequentially

isolated and sequenced in ion trap with a normalized collision energy of 35, an activation q of 0.25 and an activation time of 10 ms.

Online 2D LC Separation and LC-MS/MS analysis

The fully automated 7-cycle on-line two-dimensional LC-MS/MS was set up as described [3]. Briefly, the C18 trapping column was replaced with a biphasic precolumn (150 µm i.d.) comprised of a 3.5-cm column packed with 5 µm C18 120 Å reversed-phase material (ReproSil-Pur 120 C18-AQ, Dr. Maisch) and 3.5-cm column packed with Luna 5 µm SCX 100 Å strong cation exchange resin (Phenomenex, Torrance, CA) while all other setups remained the same. Enriched peptides were first loaded onto the biphasic precolumn. Ammonium acetate at concentrations of 0, 25, 50, 75, 125, 200 and 500 mM were then sequentially injected using a 48-well autosampler from the sample vial to elute bound peptides from precolumn to analytical column with reversed-phase separation. LC-MS/MS experiments were also performed with a 120-min linear gradient of 2-35% acetonitrile in 0.1% formic acid.

Data Processing and Analysis.

The raw data were first converted to mzXML files and DTA files using ReAdW (http://sourceforge.net/projects/sashimi/files/) and MzXML2Search (http://tools.proteomecenter.org/wiki/index.php?title=Software:MzXML2Search) programs, respectively. Bioworks 3.2 was used for protein identification by searching the DTA files against the human IPI protein database version 3.68 (87,062 entries) and its reversed complement. Initial precursor mass tolerance of 10 ppm and fragment mass deviation of 0.8 Th were set as the search criteria. The maximum number of miss-cleavages for trypsin was set as two per peptide. Cysteine carbamidomethylation was considered as a fixed modification, whereas methionine oxidation and desthiobiotinylation of lysine (+281.17394 Da) were included as variable modifications. Aside from the search parameters described above, lysine (+8 Da) and arginine (+6 Da) mass shifts introduced by heavy isotope labeling were also considered as variable modifications. The search results were then filtered with DTASelect [4] to achieve a peptide false discovery rate of 1%. Census was employed for peptide and protein quantification [5]. Extracted-ion chromatograms were first generated for peptide ions based on their *m*/*z* values and peptide intensity ratios were subsequently calculated in Census from peak areas found in each pair of extracted-ion chromatograms.

***In vitro* kinase activity assay for CDK6**

CDK6 substrate peptide EGLPTPT$_{821}$KMTPPFR derived from retinoblastoma-associated protein (Rb) was purchased from Genemed Synthesis, Inc. *In vitro* kinase assays for CDK6 are performed using mixture of 0.5 µg of CDK6/Cyclin D3 complex (SignalChem, Catalog #: C35-10H), 5 µM of CDK 6 peptide substrate EGLPT$_{821}$PTKMTPPFR, 1×Kinase Dilution Buffer III (SignalChem, Catalog #: K23-09) and 1×Kinase Assay Buffer I (SignalChem, Catalog #: K01-09). To each reaction mixture, 500 µM of ATP, GTP, or ^{S}GTP was added separately and the final volume was adjusted to 40 µL by adding H_2O. To test the inhibitory effect of ^{S}GTP on CDK6 phosphorylation, a series of ^{S}GTP solutions at concentrations of 0, 125 µM, 250 µM, 500 µM, 1 mM and 2.5 mM were added to individual CDK6 reaction mixture in the presence of a constant concentration of ATP (500 µM). *In vitro* CDK6 phosphorylation reactions were continued at 25°C for 45 min. The reactions were quenched with 10% TFA (4 µL). The

resulting peptides were further desalted by ZipTip and then analyzed by LC-MS/MS on an LTQ-Orbitrap Velos mass spectrometer.

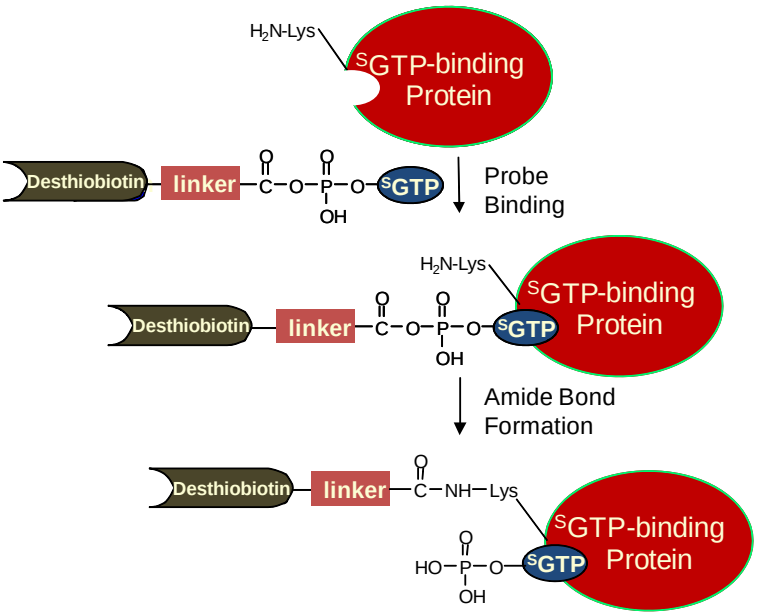
Supplementary Reference

(1) Qiu, H.; Wang, Y. *Anal. Chem.* **2007**, *79*, 5547.
(2) Olsen, J. V.; de Godoy, L. M. F.; Li, G.; Macek, B.; Mortensen, P.; Pesch, R.; Makarov, A.; Lange, O.; Horning, S.; Mann, M. *Mol. Cell. Proteomics* **2005**, *4*, 2010.
(3) Taylor, P.; Nielsen, P. A.; Trelle, M. B.; Andersen, M. B.; Vorm, O.; Moran, M. F.; Kislinger, T. *J. Proteome Res.* **2009**, *8*, 1610.
(4) Tabb, D. L.; McDonald, W. H.; Yates, J. R. *J. Proteome Res.* **2002**, *1*, 21.
(5) Park, S. K.; Venable, J. D.; Xu, T.; Yates, J. R. *Nat. Methods* **2008**, *5*, 319.

Supplementary Figures

Figure S1. (A) A schematic diagram showing the reaction between ^{S}GTP affinity probe with an ^{S}GTP-binding protein. (B) Representative of ESI-MS of purified desthiobiotin-based ^{S}GTP affinity probes.

(A)



(B)

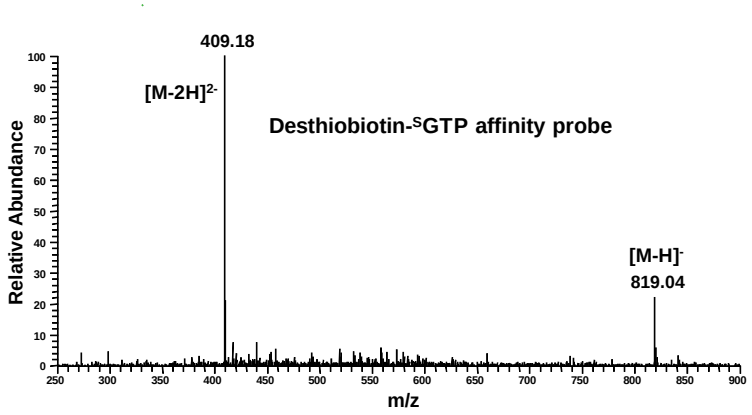


Figure S2. MS/MS of the $[M+2H]^{2+}$ ion of the phosphorylated CDK6 substrate peptide EGLPT$_P$PTKMTPPFR (*m/z*= 826.3993)

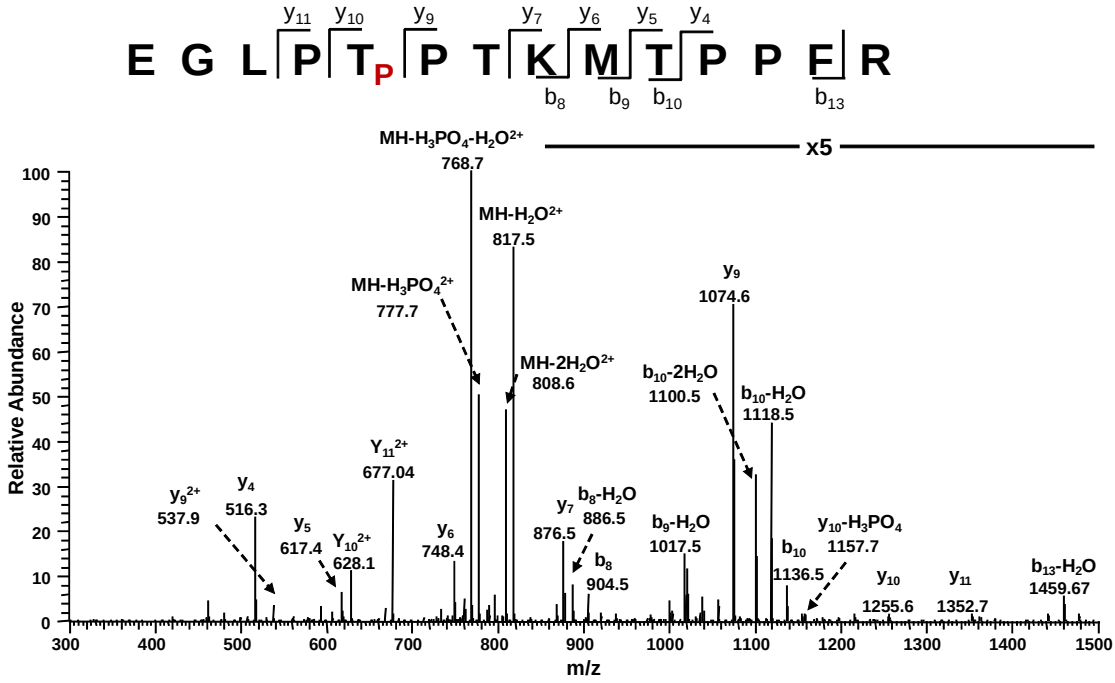


Table S1 (Shown as Excel file in online Supporting Information)**.** $R_{SGTP10/1}$ ratio in ^{S}GTP-affinity profiling experiment and $R_{SGTP/GTP}$ ratio in ^{S}GTP/GTP competition binding assay using Jurkat-T cell lysates for 199 quantified peptides from 165 ^{S}GTP-binding proteins. Those peptides with $R_{SGTP/GTP}$ ratio of "#N/A" were only quantified in ^{S}GTP-affinity profiling experiment but not in ^{S}GTP/GTP competition binding assay. GO annotations for each protein were included. The results were based on two sets of forward and two sets of reverse labeling experiments for both ^{S}GTP-affinity profiling and ^{S}GTP/GTP competition binding assay.

Table S2. Details of GO analysis of proteins with different $R_{SGTP10/1}$ ratio using Jurkat-T cell lysates.

		Total quantified protein (1925)	Possible ^{S}GTP binding protein (165)	Other quantified protein (1760)
ATP binding protein	Number	318	56	262
	percentage	16.5%	33.9%	14.9%
	p value	4.4E-33	1.5E-15	1.2E-21
	enrichment factor	1.6	3.4	1.5
GTP binding protein	Number	91	32	59
	percentage	4.7%	19.4%	3.3%
	p value	1.0E-12	8.1E-20	3.1E-4
	enrichment factor	2.0	8.4	1.4