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Identification and Validation of Inhibitor-Responsive Kinase Substrates using a New Paradigm to Measure Kinase-Specific Protein Phosphorylation Index

Xiang Li¹, Varsha Rao¹, Jin Jin¹, Bin Guan¹, Kenna L. Anderes², and Charles J. Bieberich^{1,3,*}

¹Department of Biological Sciences, University of Maryland Baltimore County, Baltimore, Maryland USA

²ApoCell, Inc. Houston, Texas USA

³Martha & Stuart Greenebaum Cancer Center, University of Maryland Baltimore, Baltimore, Maryland USA

Abstract

Regulation of all cellular processes requires dynamic regulation of protein phosphorylation. We have developed an unbiased system to globally quantify the phosphorylation index for substrates of a specific kinase by independently quantifying phosphorylated and total substrate molecules in a reverse in-gel kinase assay. Non-phosphorylated substrate molecules are first quantified in the presence and absence of a specific stimulus. Total substrate molecules are then measured after complete chemical de-phosphorylation, and a ratio of phosphorylated to total substrate is derived. To demonstrate the utility of this approach, we profiled and quantified changes in phosphorylation index for Protein Kinase CK2 substrates that respond to a small-molecule inhibitor. A broad range of inhibitor-induced changes in phosphorylation was observed in cultured cells. Differences among substrates in the kinetics of phosphorylation change were also revealed. Comparison of CK2 inhibitor-induced changes in phosphorylation in cultured cells and in mouse peripheral blood lymphocytes *in vivo* revealed distinct kinetic and depth-of-response profiles. This technology provides a new approach to facilitate functional analyses of kinase-specific phosphorylation events. This strategy can be used to dissect the role of phosphorylation in cellular events, to facilitate kinase inhibitor target validation studies, and to inform *in vivo* analyses of kinase inhibitor drug efficacy.

INTRODUCTION

Reversible protein phosphorylation regulates virtually all processes in eukaryotic cells. Protein localization, activity, and interactions are strongly influenced by the dynamic addition and removal of phosphate groups from serine, threonine, and tyrosine residues. The advent of mass spectrometry (MS)-based methods to globally survey the phosphoproteome has led to the identification of thousands of phosphopeptides in yeast as well as mammalian

*To whom correspondence should be addressed. bieberic@umbc.edu; 410 455 3125; 410 455 3875 fax.

cells lines and tissues.^{1–5} Discerning the role of protein phosphorylation in normal and pathologic processes requires quantitative measurements of shifts in phosphorylation in response to changes within the intra- and extracellular environments. The clinical importance of these measurements is difficult to overstate, given that aberrant kinase activity is associated with hundreds of diseases,^{6–9} and given the high stakes surrounding the successful development and deployment of therapeutic kinase inhibitors.^{10, 11}

Advances in MS combined with development of new technical approaches have led to rapid advances in our ability to comparatively quantify phosphopeptides in complex biological extracts.^{12–14} Proteomic strategies using stable-isotope labeling have yielded large datasets quantifying changes in phosphorylation in response to adaptive responses in cells.^{15–18} In a recent large-scale *in vivo* study using spiked-in SILAC internal standards, 10,000 phosphosites were quantified in mouse liver cells in response to insulin signaling.¹⁹ The use of stable isotope-labeled internal standards generated from cultured cells with, and without, insulin stimulation made it feasible, by comparison, to quantify changes in non-labeled mouse tissues. While comparative quantification of phosphopeptide abundance can be informative, ultimately, it is essential to measure changes in ratios of phosphorylated to total protein to obtain a detailed view of cell signaling outcomes.²⁰

In vivo, the percentage of a given protein phosphorylated at any moment can vary from <1 to nearly 100²¹, and dynamic phosphoproteome regulation is a central regulatory mechanism for cellular processes. Determining phosphorylated-to-total protein ratio has traditionally been achieved using a combination of phosphosite-specific and total protein antibodies on Western blots. New developments in stable-isotope labeling combined with high mass accuracy MS have opened new windows into the complexity of phosphorylation stoichiometry within cells.^{22, 23} In a recent study, stable-isotope labeling was used in conjunction with phosphatase treatment to quantify stoichiometries for more than 5,000 phosphorylation sites in yeast.²³ The success of MS-based methods to accurately quantify change in phosphopeptide stoichiometry provides an excellent foundation to globally map dynamic changes in phosphorylation topology. These data will clearly fill in large swaths of kinomic space and will undoubtedly lead to insights that can be exploited to dissect the complex interconnections that characterize cellular signaling pathways. Ultimately, however, a robust understanding of phosphorylation dynamics must not only address which phosphorylation sites are altered in disease states or adaptive responses, but also which kinases and phosphatases mediate the observed changes.^{20, 24–26} These data will be crucial to develop targeted therapies to block specific phosphorylation or de-phosphorylation events that play causal roles in disease, but are unlikely to emerge from shotgun proteomic studies.

By necessity, analyses of kinase-specific phosphorylation dynamics will require deep knowledge of the true physiological substrates of all kinases and their associated phosphatases. Although the importance of this body of knowledge has long been recognized, limited progress has been made to comprehensively map these relationships across the kinome. In a recent global analysis, an array of phosphosite-specific antibodies was used in concert with SILAC (Stable Isotope Labeling with Amino acids in Cell culture) to quantify phosphorylation changes in Ataxia Telangiectasia Mutated kinase (ATM) and Ataxia Telangiectasia and Rad-3-related kinase (ATR) substrates by immunoprecipitation in

response to gamma irradiation.²⁷ This powerful approach revealed more than 900 DNA damage-regulated phosphosites. Although this method has considerable merit, it does not account for changes in protein abundance, a factor that has recently been recognized as critical for comprehensively quantifying dynamic changes in phosphopeptide abundance.²³ There is clearly a need to develop robust technologies to quantify kinase-specific changes in substrate phosphorylation. The need is most urgent in the clinical arena, where kinase inhibition has emerged as a predominant new strategy to treat an array of pathologic conditions, particularly neoplastic disease.²⁸

The pharmaceutical industry has invested heavily in searching for kinase-specific inhibitors for anticancer therapy.¹¹ However, the success rate for these highly anticipated new agents has been disappointingly low, with multiple late-stage, high profile failures.^{29–32} Lack of success in identifying pharmacodynamic (PD) biomarkers is considered to be one of the most critical elements in improving success rates for targeted therapies.^{28, 33, 34} Incorporating validated PD biomarkers into the drug development pipeline can play a critical role in making go/no-go decisions, and in determining optimal dose and scheduling of efficacious compounds.³³ Judicious use of PD biomarkers has the potential to dramatically reduce drug development costs and time from lead compound identification to clinical deployment. Since kinase inhibition is the intended mode of action for most molecularly targeted compounds currently under development¹¹, the identification of proximal PD biomarkers, ideally, direct kinase substrates that respond to inhibitor treatment, is critical.³⁵ Despite recent technical advances in quantitative assessment of the phosphoproteome,³⁶ validated PD biomarkers for most oncogenic kinases are not known. Determining *in vivo* efficacy of these compounds requires knowledge of physiological kinase substrates and tools to monitor their phosphorylation status.

In this study, we present an activity-based paradigm to measure kinase-specific substrate phosphorylation stoichiometry *in vivo*, and apply this strategy to profile substrates that respond *in vivo* to a small molecule kinase inhibitor. The approach combines the Reverse In-gel Kinase Assay (RIKA)³⁷ with a refined non-enzymatic method for complete proteome de-phosphorylation.³⁸ Non-phosphorylated substrate molecules are first quantified in the presence and absence of a specific kinase inhibitor. Total substrate molecules are then measured after complete chemical de-phosphorylation, and a ratio of phosphorylated-to-total substrate is derived. Using this novel strategy to measure kinase inhibitor-induced, kinase-specific phosphorylation index changes, we identified substrates with highly desirable PD biomarkers properties.

EXPERIMENTAL SECTION

Reverse in-gel kinase assay

To prepare each 10 ml RIKA SDS-PAGE gel, the following were combined: 3.5 ml kinase (10–1000 μ g in 8 M urea, 100 mM NaH₂PO₄ (pH 8.0), 250 mM imidazole), 2.5 ml of 0.5 M Tris (pH 8.8), 4 ml 30% acrylamide/bis 37.5:1 (Bio-Rad), 50 μ l 10% ammonium persulfate, 50 μ l 20% SDS and 10 μ l TEMED. After electrophoresis, RIKA gels were washed twice for 30 minutes each with 200 ml 20% isopropanol, 50 mM Tris (pH 7.5) to remove SDS, then with 50 mM Tris (pH 7.5), 2 mM β -mercaptoethanol twice for 30 minutes each at room

temperature (18–24 °C). Gels were then incubated in 6 M urea, 20 mM Tris (pH 7.5), 20 mM MgCl₂ twice for 15 minutes each to denature proteins. Gels were then carried through a descending urea gradient (6 M, 3 M, 1.5 M, 0.75 M) at 4 °C by replacing half the buffer in the container with refolding buffer (50 mM Tris pH 7.5, 2 mM β-mercaptoethanol, 0.05% Tween 20) at 15 minute intervals. After incubating in refolding buffer three times for 15 minutes each, followed by overnight incubation at 4 °C, gels were incubated in 200 ml 20 mM Tris (pH 7.5), 20 mM MgCl₂ for 30 minutes at room temperature. In-gel kinase reactions were performed at room temperature in 20 mM Tris (pH 7.5), 20 mM MgCl₂, 2 mM DTT containing 2.5 μCi γ-³²P-ATP/ml (9,000 Ci/mmol; Perkin Elmer) in a total volume of 200 μl. To stop the reaction and remove unlabeled γ-³²P-ATP, gels were washed twice with 5% TCA, 1% NaH₂PO₄, twice, and with ultra-pure H₂O until no isotope was detectable in the buffer using a hand-held Geiger counter. Autoradiograms were obtained from dried gels. CK2 concentration in CK2 RIKAs was 5 μg/ml, and PKA concentration was 20 μg/ml.

HF treatment of total cell lysates

HeLa cells were resuspended in 50 mM ammonium bicarbonate, 0.5% NP40 containing a protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). The suspension was agitated at 4 °C for 30 minutes to lyse the cells. The suspension was centrifuged at ~13,000 g for 10 minutes in a refrigerated microcentrifuge to remove cell debris. The supernatant was then lyophilized. Lyophilized protein pellets were washed 3 times with –20°C acetone containing 20 mM DTT to remove salts. Acetone was completely removed by speed vacuum. To de-phosphorylate proteins, dried pellets were completely re-dissolved in 100 μl of 60% HF (Sigma-Aldrich, St. Louis, MO) and incubated on ice for 90 minutes. The 60% HF solution was prepared by mixing six volumes of 70% HF-pyridine with one volume of 8M urea, 100 mM NaH₂PO₄, pH 7.5. To stop the de-phosphorylation reaction, 900 μl ice-cold ultrapure H₂O was added to the protein solution. The mixture was then lyophilized. The lyophilizer was equipped with a soda lime trap to capture evolved HF. To remove salts, the protein pellet was washed with 900 μl 6% TCA and 20 μl 2% deoxycholate, vortexed, and incubated on ice for 30 minutes. The suspension was then centrifuged and washed 4 times with –20 °C acetone to remove TCA. Acetone was completely removed by speed vacuum.

Affinity pull-down

HeLa cells were cultured in DMEM supplemented with 10% FBS in a humidified incubator at 37 °C. HeLa cells at 90% confluence in a 10 cm dish were transfected with 8 μg of a plasmid consisting of the complete human EF-1β open reading frame cloned in pcDNA3.1 using LipoD293TM (SigmaGen, Ijamsville, MD) transfection reagent following the manufacturer's recommendations. Media was replaced 12 hours after transfection. Transfected cells were treated with CX-4945 (diluted from a 100 mM stock in Na₂HPO₄) in RPMI at various concentrations for specific exposure times beginning 24 hours post-transfection. Cells were harvested using 0.25% trypsin/EDTA, washed with 1X PBS, and lysed in 1 ml 6M guanidine, 100 mM NaH₂PO₄ (pH 7.5), 20 mM imidazole, 1% Triton X-100, 1% Tween-20. Lysates were centrifuged at ~13,000 g for 10 minutes in a microcentrifuge to remove cell debris. 300 μl of Ni-NTA agarose beads (600 μl total slurry,

Qiagen, Valencia CA) equilibrated with the same buffer was added to the lysates. The mixture was incubated for one hour with agitation to capture the his-tagged protein on the beads. The mixture was transferred to a mini spin column (Natrix Separations, Ontario, Canada), and the lysates were separated from the beads by centrifugation at 1,000 g for 1 minute in a microcentrifuge. The beads were washed with 600 μ l lysis buffer four times to remove nonspecific proteins. Beads were then washed twice with 600 μ l 8 M urea, 100 mM NaH_2PO_4 (pH7.5), 20 mM imidazole twice, and the his-tagged protein was then eluted with 400 μ l 8 M urea, 100 mM NaH_2PO_4 (pH7.5), 250 mM imidazole twice (E1 and E2).

HF treatment of pull-downs

200 μ l of E1 was transferred to a fresh microcentrifuge tube and 30 μ l of a slurry containing 50% CaF_2 beads (Sigma-Aldrich, St. Louis MO) was added. The protein was precipitated by adding 900 μ l of 6% TCA and 20 μ l of 2% sodium deoxycholate to the solution. The mixture was vortexed briefly and incubated on ice for at least 30 minutes, then centrifuged for 10 minutes to pellet the proteins. The precipitated proteins and CaF_2 beads were washed twice with -20°C acetone, 20 mM DTT to remove trace TCA. Acetone was removed by air drying or speed vacuum. Immediately before HF treatment, a 70% HF-pyridine stock solution was diluted to 60% by mixing 6 volumes HF-pyridine with one volume of 8M urea, 100 mM NaH_2PO_4 , 20 mM imidazole. The 60% HF solution was cooled to 0°C , and 100 μ l was added to the CaF_2 bead/protein pellet, and the mixture was vortexed for 30 seconds to resuspend the protein then incubated on ice for 90 minutes. To remove HF, 900 μ l 6% TCA and 20 μ l 2% sodium deoxycholate was added, the mixture was vortexed briefly then incubated on ice for 30 minutes, then centrifuged for 10 minutes to pellet the proteins. The pellet was washed 4 times with 800 μ l -20°C acetone. 200 μ l of 2X Laemmli buffer and 100 μ l 1M DTT was added to the pellet, and protein was re-solubilized by vortexing continuously for 30 minutes.

Determination of I^p and I^{dp} using RIKA

The I^{dp} for a completely dephosphorylated protein is defined as 1.0. The de-phosphorylation index of a given protein sample is derived by comparing the RIKA signal intensity of the protein with the RIKA signal intensity of the same sample after complete de-phosphorylation in a parallel RIKA assay: $\text{Intensity of subject sample} / \text{intensity of de-phosphorylated sample}$. De-phosphorylation was accomplished by treating the subject protein sample with HF solution. The variation of protein abundance in the process of HF treatment was normalized using specific internal controls. In the experiment shown in Figure 2, the variation in TEBP abundance was normalized with another CK2 substrate, Acidic leucine-rich nuclear phosphoprotein 32 family member B (ANP32B), which is non-phosphorylated prior to the HF treatment. Any change in ANP32B RIKA signal intensity after HF treatment is due to change in protein abundance only, not to change in I^p . In contrast, the change in RIKA signal intensity for TEBP can be caused by a change in both protein abundance and I^{dp} . Protein abundance change (technical protein loss) for both TEBP and ANP32B are assumed to be the same. The I^{dp} of TEBP is derived by normalizing the change in RIKA signal with the change in protein abundance before and after HF treatment. The change in protein abundance in the experiment shown in Figure 3 (2D RIKA using whole cell protein extract), was normalized using internal CK2 substrates that are not

phosphorylated *in vivo* (GP94 in mouse PBMCs and PP2C in HeLa lysates). The change in protein abundance in the experiment shown in Figure 5 (affinity pull-down), was normalized by measuring the protein abundance using Western blot. On rare occasions, the experimentally derived I^{dp} value exceeded 1.0 due to technical variance, but was never greater than 1.12. In these cases, the I^{dp} value was set to 1.0 in calculations. The I^p in each circumstance was calculated by subtracting I^{dp} from 1.0.

Identification CK2 substrates by LC-MS/MS

Spots on RIK-A gels containing potential CK2 substrates were aligned and excised as illustrated in Figure S1. Gel pieces were rehydrated in 200 μ l H₂O in a petri dish. The cellophane membrane was removed from the gel piece and the gel was washed again with 200 μ l H₂O and transferred to a 0.7 ml micro-centrifuge tube. The gel was broken into 4–5 small pieces with a pair of fine-tipped forceps. 200 μ l ACN was added and the tube was incubated for 5 minutes at room temperature. The ACN was removed completely and 30 μ l of MS-grade trypsin (Trypsin Gold, Promega) dissolved in 50 mM ammonium bicarbonate, 10% ACN to achieve a final trypsin concentration of 10–20 ng/ml was added to each tube. The tube was then placed on ice for one hour to allow trypsin penetration, then transferred to a 37°C shaker and incubated for 3 hours to overnight for digestion. 100 μ l 2% formic acid was added to the digestion mixture and incubated with vigorous agitation for 10 minutes to extract digested peptides. The first extraction solution was transferred to a new micro-centrifuge tube, and gel pieces were extracted with 100 μ l 1% formic acid, 50% ACN for 20 minutes, twice. The second and third extraction was pooled with the first. The gel was further extracted with 100% ACN for 10 minutes and pooled with the previous extractions. The extracted peptide solution was lyophilized and resuspended in 12 μ l of 0.5% formic acid. 10 μ l of the solution was applied to a C18 column coupled on-line to a ThermoFisher LTQ linear ion trap mass spectrometer and eluted with a formic acid/ACN gradient. MS analysis was done in unattended data-dependent mode, with the mass spectrometer switching between acquiring full scan mode survey mass spectrum and consecutive fragmentation of the top five most abundant ions using CID and ETD. The data were collected using XCalibur (Thermo XCalibur 2.1.0.1139, released on February 03, 2009, ThermoFisher).

Database Searching and Criteria

Acquired data (Raw files) were analyzed using the Sequest algorithm (Thermo Proteome Discoverer 1.1.0263, released in 2009, ThermoFisher) by searching human Fasta database (60090 sequences, 26170332 residues) or mouse Fasta database (53788 sequences, 25043435 residues) under conditions as following: trypsin specificity; peptide mass tolerance set to 0.8 Da; fragment mass tolerance at 0.8 Da; a maximum of two allowed missed cleavages; carbamidomethyl, deamidation and methionine oxidation as the variable modifications peptides from keratins and CK2 are excluded as known contaminants. Potential protein identities were filtered according to Xcorr score. Proteins with a minimum of 3 peptides, each meeting the following criteria, were considered as high confidence hits: Xcorr Score > 2 for singly charged peptide; Xcorr Score > 2.2 for double charged peptide;³⁹ CID probability score > 10 or ETD probability score > 1. The identities of the proteins were further confirmed by matching the apparent molecular weight and pI to the predicted values.

Protein IEF

Protein lysates were precipitated with TCA (Sigma-Aldrich, St. Louis, MO) as described above. Protein pellets were resuspended in 7 M urea, 2 M thiourea, 1% C7BZO (Sigma-Aldrich, St. Louis, MO), 50 mM DTT, 1% IEF buffer (GE Health, Piscataway, NJ). Lysates were then applied to an immobilized pH gradient (IPG) strip and focused on an IEF cell (Bio-Rad Laboratories, Hercules, CA). Ampholytes of the same pH range were added to a final concentration of 0.5%. No additional ampholytes were added when pH 3–11 IPG strips were used.

Purification of His-tagged proteins

His-tagged proteins were purified using nickel chromatography under standard conditions. Recombinant CK2 and PKA were expressed under native conditions, and purified under denaturing conditions. Briefly, the kinases were induced with IPTG at room temperature (for PKA), or 37 °C (for CK2). The cells are harvested, resuspended in lysis buffer, and lysed using a French press. The lysates were centrifuged at 20,000 g for 30 minutes, and pellets were discarded. The supernatant was incubated with equilibrated nickel-NTA resin to capture his-tagged recombinant kinases. The resin was washed once with 6M Guanidine-HCl, 100 mM Na₂HPO₄ (pH 7.5), 20 mM imidazole, pH 7.5, twice with 8M Urea, 100 mM Na₂HPO₄ (pH 7.5), 20 mM imidazole, pH 7.5, and eluted with 8 M Urea, 100 mM Na₂HPO₄ (pH 7.5), 250 mM imidazole, pH 7.5. Other recombinant proteins, including TEBP, ANP32B, Purine Rich Protein B (PURB), and EF-1 β , were purified under native conditions.

In vitro kinase assays of recombinant CK2 α substrates

Recombinant putative CK2 α substrates purified from *E. coli* were dialyzed against 150 mM NaCl, 20 mM Tris, pH 7.5 prior to in vitro kinase assay. To label a recombinant protein by CK2 α , ~5–10 μ g of each recombinant protein was incubated with 0.5 μ g CK2 α at room temperature for 60 minutes in a 1X CK2 α reaction buffer (New England Biolab, Beverly, MA) in the presence of 30 nM γ -³²P ATP and 200 μ M cold ATP. The reactions were stopped by adding an equal volume of 2X Laemmli buffer. The reaction mixture was resolved by SDS-PAGE and transferred to a PVDF membrane. The PVDF membrane was stained with Coomassie brilliant blue and dried prior to autoradiography.

Western Blot

Western blot analyses followed standard protocols. Anti-HA rat monoclonal antibody (clone 3F10, Roche Applied Sciences) was used at a final concentration of 50 ng/ml. Rabbit polyclonal anti-CK2 antibodies were a gift from Dr. David Litchfield, and were diluted 1:5000.

Image quantification for Western blot and RIKa signals

Western blot and RIKa autoradiograms were scanned using an Epson Expression 1600 scanner, and image files were saved in TIFF format. The signals were quantified using ImageJ (ImageJ 1.42q, National Institute of Health). Background subtraction was performed

by selecting blank areas in close proximity to signal. The data were analyzed using Microsoft Excel.

Treatment of mice with CX-4945

Animal care was provided in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Procedures using mice were approved by the UMBC Institutional Animal Care and Use Committee. CX-4945 at various concentrations dissolved in 12.5 mM Na₂HPO₄ was administered to ~8 week-old FVB/N female mice in a 100 µL volume by tail vein injection. Pools of four animals were euthanized and total blood was collected by cardiac puncture. RBCs were lysed using Gey's solution and PBMC total protein lysates were prepared and analyzed by 2D RIKA.

RESULTS

Developing a RIKA-based method to measure kinase-specific substrate phosphorylation index

The RIKA is a robust method for detecting physiological kinase substrates in complex protein extracts.³⁷ In this assay, kinase substrates are radioactively labeled by a kinase polymerized in an SDS-PAGE gel. The RIKA detects phosphoacceptor sites on substrate proteins that are unoccupied when the extract is made: a phosphorylation site already occupied *in vivo* cannot accept a phosphate group during the assay, and is therefore not detected. Hence, only the non-phosphorylated fraction of a substrate pool can be detected and quantified in a RIKA. To extend its utility, we further developed the assay to measure the ratio of de-phosphorylated to total protein (which we hereafter refer to as the de-phosphorylation index, I^{dp}) of detectable substrates. Subtracting the I^{dp} value from one yields the canonical substrate phosphorylation index, I^p . In a RIKA, the non-phosphorylated pool of each substrate is detected as signal on an autoradiogram (Figure 1A,B). When the phosphorylated to non-phosphorylated balance is shifted, for example, when a kinase inhibitor is present, the RIKA signal increases proportionately (Figure 1C,D), providing insights into inhibitor activity toward specific substrates. However, to quantify changes in I^p , it is necessary to simultaneously measure the total pool of each substrate within the extract. This could be accomplished under conditions where complete protein de-phosphorylation is achieved prior to the assay (Figure 1E,F).

To develop a method to quantitatively de-phosphorylate proteins in a complex extract, we adapted an approach used to strip phosphate groups from synthetic peptides without detectable damage.³⁸ HeLa cells were metabolically labeled using ³²P-orthophosphate, and protein extracts were treated with hydrofluoric acid (HF) in urea on ice. Essentially complete de-phosphorylation of *in vivo*-phosphorylated proteins was achieved within 2 hours of HF exposure (Figure 2A). No change in protein integrity after HF treatment was detected by Coomassie Blue staining after 1D SDS-PAGE (Figure 2B), nor by 2D separation and silver staining (Figure S2, Supporting Information). To determine whether HF-treated proteins can be quantitatively re-phosphorylated, the CK2 substrate Telomerase Binding Protein (TEBP) was phosphorylated *in vitro* under conditions favoring quantitative substrate phosphorylation, HF treated, then re-phosphorylated in a RIKA. To demonstrate

that TEBP was phosphorylated to near completion, the reaction was analyzed by SDS-PAGE and transferred to a PVDF membrane. A single species with a clear molecular weight shift was observed after CK2 phosphorylation of TEBP, suggesting that phosphorylation was complete (Figure S3, Supporting Information). As expected, *in vitro* CK2 phosphorylation of TEBP before the assay precludes its detection in a CK2 RIKA. The extent of re-phosphorylation was measured semi-quantitatively by analyzing the RIKA signal with and without HF-treatment (*cf.* Figure 2B, lanes 1 & 5), and normalizing to the silver stain signal. Ninety-five percent of the signal could be restored (Figure 2B). These data demonstrate that essentially complete protein de-phosphorylation is achieved by HF exposure while phosphoacceptor site integrity is maintained. To demonstrate that the RIKA can be quantitative, a titration of TEBP was analyzed in a CK2-containing gel. The assay was quantitative in a range of <1 ng to ~10 ng TEBP (Figure S4, Supporting Information). Next, we showed that the HF-RIKA approach could accurately determine the phosphorylation index of a substrate pool with known phosphorylation index (Figure 2C). Fully *in vitro* phosphorylated TEBP was mixed with mock-phosphorylated TEBP to create substrate pools with I^p varying from 1.0 (completely phosphorylated) to 0.5 (50% phosphorylated). A separate non-phosphorylated CK2 substrate³⁷ (ANP32B) was included as an internal technical control. After HF treatment, an experimental I^p was determined in CK2 RIKAs. The accuracy of experimentally determined I^p ranged from 92% to 98%, demonstrating that this method can reliably detect a ~10% shift in I^p (Figure 2D). It is important to note that TEBP is known to be phosphorylated by CK2 on two sites⁴⁰. Thus, the I^p values measured represent the average combined phosphosite occupancy.

Validation of phosphorylation indexing by RIKA in cultured cells

To validate that our method can measure the phosphorylation index of kinase substrates *in vivo*, we tested it using HeLa cells treated with CK2 inhibitor. CX-4945 is a small molecule CK2 inhibitor currently in clinical trials to treat solid tumors and Castleman's disease.⁴¹ HeLa cells were drug-exposed for 4 hours, and cell lysates treated with HF solution to dephosphorylate the proteins, and the lysates were analyzed in 2D CK2 RIKAs. The signals were quantified and the phosphorylation index of CK2 substrates were calculated for lysates before and after inhibitor treatment. Some CK2 substrates were also identified by LC-MS/MS (Figure S5, Table S1, Supporting Information). Most CK2 substrates have a high phosphorylation index in the control cell lysates, which concurs with the fact that CK2 is a constitutively active kinase.⁴² Nearly all substrates showed an obvious decrease in phosphorylation index upon CX-4945 treatment, however the extent of change in I^p was highly variable (Figure 3A, Table 1). For example, the I^p of Elongation Factor-1 β (EF-1 β) decreased from 0.97 to 0.04 in the presence of CX-4945 (Table 1). In contrast, the I^p of 60S Ribosome Protein P2 (RP2) changed marginally, from 0.95 to 0.75. To demonstrate that CX-4945-induced phosphorylation index decrease is specific, we treated HeLa cells with CX-4945 at increasing dosages, and at various time points. The effects of increasing CX-4945 dosage on CK2 substrate hypo-phosphorylation were also readily detected using RIKA (Figure 3B). Hypo-phosphorylation was evident 15 minutes after drug exposure, and continued to increase during the 6-hour experiment, reflecting the fact that an effective concentration of CX-4945 was maintained in the culture media (Figure 3C). Other CK2 inhibitors, for example 4,5,6,7-tetrabromobenzotriazole (TBB) (Figure S6, Supporting

Information), and 5,6-dichloro-1-D-ribofuranosylbenzimidazole (DRB)(data not shown) showed similar dose- and time-dependent effects in LNCaP cells.

To further confirm that the proteins responding to CX-4945 exposure are *in vivo* CK2 substrates, and that CX-4945 is blocking CK2 activity, we knocked down CK2 using siRNA. The lysates were HF-treated and analyzed on 2D CK2 RIKAs. The overall pattern of CK2 substrate hypo-phosphorylation was similar in siRNA- and CX-4945-treated cells, however the extent of substrate response varied (cf. Figure 3A, Figure S5, Supporting Information).

Further validation by affinity pull-down

To confirm that the signal changes observed in 2D CK2 RIKAs are due to the change in phosphorylation index of CK2 substrates and not co-migrating proteins, we developed a validation approach using transient expression of CK2 substrates in HeLa cells. Based on the experiments described above (Figure 3), EF-1 β appeared to respond dramatically to CK2 inhibition by exhibiting rapid, extensive, and sustained de-phosphorylation upon CX-4945 exposure. EF-1 β has also been previously reported as a potential CK2 substrate.⁴³ To confirm that the change in signal intensity is due to changes in EF-1 β phosphorylation index, His+HA-tagged EF-1 β was transiently expressed in HeLa cells, and pulled down by nickel affinity chromatography after exposure of cells to CX-4945. The abundance of EF-1 β was measured by Western blot using anti-HA antibody, and the phosphorylation index was calculated. Hypo-phosphorylation of EF-1 β was detected within 10 minutes of drug exposure that increased with both time and dosage (Figure 4A, B; Figure S8A & B, Supporting Information). These data confirm that EF-1 β can be directly phosphorylated by CK2 in HeLa cells, and confirm that EF-1 β has a high *in vivo* I^p (Table 1) that undergoes a rapid and profound change in response to CX-4945. The transiently expressed EF-1 β completely mimicked the kinetics of endogenous EF-1 β upon CK2 inhibition.

To further confirm that the change of signal of EF-1 β is due to CK2 phosphorylation, we determined the site of CK2 phosphorylation in EF-1 β . Recombinant EF-1 β was phosphorylated to completion *in vitro* (and tryptic digests of the resulting products were analyzed by LC-MS/MS. Analysis the resulting spectra revealed that CK2 phosphorylated EF-1 β on serine 106 (S106) (Figure S9A, Supporting Information). EF-1 β S106 was confirmed as a CK2 phosphoacceptor site by alanine mutagenesis (S106A), which nearly abolished the ability of CK2 to phosphorylate EF-1 β *in vitro* (Figure S9B, Supporting Information). Expression and affinity pull-down of His+HA-tagged EF-1 β ^{S106A} in HeLa cells followed by analysis in CK2 RIKAs demonstrated that EF-1 β ^{S106A} was not phosphorylated in HeLa cells (Figure S9C, Supporting Information). Taken together, these data validate that the observed signal changes in CK2 2D RIKAs are due to changes of CK2 substrate phosphorylation index on CK2 phosphorylation sites.

In contrast to the extensive change of EF-1 β phosphorylation index, the response of Nucleophosmin 1, which also has a high *in vivo* I^p , appeared to be marginal (Figure 3A). To directly compare time and dose responsiveness of EF-1 β and Nucleophosmin 1 to CX-4945, the tagged proteins were co-expressed in HeLa cells, and their phosphorylation index was measured. EF-1 β showed a sharp increase in I^{dp} , while Nucleophosmin showed only a ~2

fold change (Figure 4C; Figure S8C, Supporting Information), highlighting the differential responsiveness of CK2 substrates to CX-4945 treatment.

Both EF-1 β and Nucleophosmin have a high I^p in HeLa cells. To determine whether this approach is also valid to measure changes of phosphorylation index of kinase substrates with a lower *in vivo* I^p , Protein Disulfide Isomerase Associated 6 (PDIA6) was analyzed by the same strategy. A change in PDIA6 I^p was also readily detected (Figure. 4D; Figure S8D, Supporting Information).

Using RIKA-based phosphorylation indexing to measure kinase inhibitor efficacy in animals

One of the most important applications of phosphorylation index measurement is to test the efficacy of kinase inhibitors in patient samples. Although CX-4945 efficaciously inhibited CK2 activity in HeLa cells, we sought to determine its efficacy in a preclinical animal model. To analyze the response to CX-4945 treatment in a whole animal, the I^p s of CK2 substrates in protein extracts of peripheral blood mononuclear cells (PBMCs) harvested from drug-treated mice were analyzed, and several responding proteins were identified by LC-MS/MS (Figure S10, Supporting Information). Decreased CK2 substrate phosphorylation was readily detected upon CX-4945 administration in a dose dependent manner (Figure S11, Supporting Information). To demonstrate the specificity of the CX-4945 response, a Protein Kinase A (PKA) RIKA was performed on treated and mock-treated PBMC lysates. No hypo-phosphorylation of PKA substrates was evident upon CX-4945 exposure (Figure S10, Supporting Information).

In contrast to HeLa cells where nearly all CK2 substrates responded, phosphorylation indexing revealed that in PBMCs, a robust response was limited to a subset of CK2 substrates (Figure 5). Changes in phosphorylation index varied greatly among PBMC responder proteins. Some substrates, for example, Purine Rich Protein B (PURB) and Tubulin β showed extensive de-phosphorylation while others changed minimally (Figure 5). Remarkably, several substrates with a high *in vivo* I^p , including Nucleophosmin 1 and 2 and Nascent Polypeptide Binding Protein, showed no measurable response to CX-4945 treatment (Figure 5, circles) despite the fact that these substrates responded well in HeLa cells. These data demonstrate the utility of this technology to monitor kinase inhibitor efficacy in a preclinical model, and highlight the potential limitations of using cultured cells to characterize PD biomarkers. In total, 16 CK2 substrates that responded to CX-4945 treatment in HeLa cells were identified in CK2 RIKAs (Figure S5, Supporting Information). The majority (14/16) were also identified in CK2 RIKA gels using mouse PBMC lysates (Tables S1 & S2; Figures S5 & S10, Supporting Information). However, substrate responses differed significantly in HeLa cells and PBMCs. Overall, only 5 of the 14 overlapping substrates showed a measurable response in PBMCs (Tables S1, 2, Supporting Information).

Using RIKA to quantify PD effects of CX-4945 treatment

A critical aspect of PD biomarkers is their ability to report on drug efficacy over time and at varying doses. Dynamic regulation of protein phosphorylation is complex, and is dependent on kinase activity, substrate localization, access to phosphatase activity, and other factors.

Determining which kinase substrates have suitable PD characteristics must therefore be determined empirically. To demonstrate the utility our approach to identify suitable PD CK2 inhibition biomarkers in a readily assayable tissue, PBMCs harvested from pools of mice at various time-points after CX-4945 administration were analyzed in CK2 RIKAs. Analysis of the hypo-phosphorylation kinetics of four responsive CK2 substrates revealed clear differences in response kinetics (Figure 6). Hypo-phosphorylation of Rp2 peaked at 15 minutes, Purb at 30 minutes, and Ef-1 β and Suppression of Tumorigenicity 13 at 1 hour after intravenous dosing. However, the effect was transient, and all substrates were mostly re-phosphorylated within five hours, albeit with variable kinetics (Figure 6). These data are consistent with CX-4945 PK data, and reflect clearance of the drug over time⁴⁴.

Validation of a new CK2 substrate by affinity pull-down and RIKA

For many clinically relevant kinases, the majority of their physiological substrates are unknown,⁴⁵ To demonstrate the utility of RIKA-based phosphorylation indexing for establishing the *bona fides* of previously unknown kinase substrates as true *in vivo* substrates, the transcription factor PURB was His+HA-tagged, expressed in HeLa cells, and characterized using the pull-down/CK2 RIKA strategy. The results showed that PURB is indeed phosphorylated by CK2 *in vivo* (Figure S13B, C, Supporting Information), and responds to CX-4945 treatment. As expected, PURB was also robustly phosphorylated by CK2 *in vitro* (Figure S13A, Supporting Information).

DISCUSSION

The biological effects of protein kinase and phosphatase activity can be far-reaching and highly complex, involving direct substrates, and, through cross-talk with other kinases, a vast array of other proteins.^{46, 47} Shotgun strategy-based proteomic studies have begun to extensively catalog phosphorylation changes that occur in response to specific stimuli. In recent studies, combining high mass accuracy MS with SILAC has enabled unparalleled opportunities to quantify phosphopeptide abundance. However, determining how these changes in abundance are directly or indirectly connected to the activity of *specific* kinases has not been systematically addressed.^{20, 26} Yet these data are required to support the development of therapeutic approaches to intervene when aberrant kinase activity is associated with disease. The approach we report here addresses this need by revealing *direct* physiological protein kinase substrates that become hypo-phosphorylated in response to kinase inhibition. This method

Large-scale phosphoproteomic methods have been developed to measure the effects of specific kinase inhibitors in cultured cells, but cannot distinguish direct from indirect changes in phosphorylation.^{48–51} In these proteome-wide studies, decreased phosphorylation for many peptides in the presence of inhibitors has provided insights to evaluate kinase inhibitor efficacy. The fact that many phosphorylation events detected in large-scale studies are likely indirect^{47, 52} is due in part to the lack of specificity of ATP analog-based protein kinase inhibitors.¹⁰ Responding peptides could be substrates of the intended target, or of off-target kinases. Determining specificity of responding peptides can be further clouded by the fact that the phosphoproteome is regulated by an integrated network of kinases whose

activity is capable of generating inordinate complexity: when the activity of one kinase is affected, the activity of many other kinases can be affected through cross-regulation. Computational methods can leverage the power of large-scale, MS-based strategies to predict kinase-substrate relationships, however, it is essential to independently confirm these predictions experimentally.^{53, 54} In the absence of information on specific kinase-substrate relationships, it is often not clear how deeply the intended inhibitor target is affected. In contrast, the method we describe provides data on which direct physiological substrates are responding, and to what extent. The availability of such data are becoming increasingly important as regulatory agencies place emphasis on demonstrating that investigational new drugs are hitting their *intended* targets *in vivo*.⁵⁵

To achieve the goal of identifying direct kinase substrates, our approach relies on the use of 2D-gel electrophoresis, despite its limited sensitivity in protein detection compared to purely MS-based strategies. Without prior enrichment by chromatographic or other methods, analyses of whole cell extracts will be likely to yield insights into the inhibitor response of relatively abundant kinase substrates. However, in terms of biomarker identification, this may not be a limitation, but rather, an advantage, since high abundance proteins are more desirable due to ease of detection. Moreover, when coupled with high-resolution fractionation, the sensitivity of RIKA-based kinase substrate analyses can be increased dramatically. For example, we envisage that affinity pull-down of specific protein complexes from cultured cells or animal tissues in the presence and absence of inhibitors will allow us to probe the effects phosphorylation changes on complex assembly, disassembly, and function. The advantage of using our approach over MS-based methods after pull-down is the ability to relate phosphorylation changes to the specific kinase responsible *in vivo*.

A novel 2D gel –based strategy for identifying inhibitor-responsive kinase substrates was recently reported in an elegant functional proteomics study to identify CK2 substrates responding to small molecule inhibitors in cultured cells. Gyenis et al.⁵⁶ developed a strategy combining metabolic labeling with ³²P-orthophosphate with transfection of inhibitor-resistant CK2 mutants to identify and validate inhibitor-responsive proteins as true physiological substrates. Interestingly, the translation factor EF-1- δ was shown to become hypo-phosphorylated on S162 in response to the CK2 inhibitor TBBz. Using a cross-reactive phospho-specific antibody developed against EF-1- δ , EF1- β was also observed to respond to TBBz. Our studies independently identified the orthologous serine residue (S106) in EF1- β as responsive to CX-4945, in good agreement with these data. While the approach developed by Gyenis et al.⁵⁶ can readily detect inhibitor-responsive kinase substrates in cultured cells, it would be difficult to apply to pre-clinical animal models and is not amenable to analysis of patient samples.

Systematic analysis of CX-4945-induced changes in phosphorylation dynamics among CK2 substrates revealed a broad range of responses. Some substrates, exemplified by EF-1 β and PURB, underwent rapid and extensive changes in phosphorylation, whereas Nucleophosmin, a well-established CK2 substrate, showed a marginal response. Other substrates, for example PDIA6, responded moderately. These data likely reflect the overall complexity of regulatory influences that affect protein phosphorylation status. For example,

some substrates (i.e., EF-1 β) may be rapidly de-phosphorylated when CK2 is inhibited due to the proximity of a phosphatase, whereas others (i.e. Nucleophosmin) may be protected from phosphatase activity by virtue of their cellular location or presence within a complex. Our data suggest that both PURB and EF-1 β are good candidates for CK2 inhibitor PD biomarkers.

Application of our method to measure kinase-specific phosphorylation changes that occur in response to a variety of conditions can significantly extend the current boundaries of kinomic informatics. This information is critical to support efforts to address one of the fundamental, largely unaddressed questions in kinomics: What are the functional consequences of each protein phosphorylation event?^{20, 24, 25} Elucidating the function of phosphorylation is technically demanding, and, by necessity, is predicated upon establishing unequivocal connections between kinases and their physiological substrates. Identification of direct kinase substrates that are rapidly de-phosphorylated upon kinase inhibition may reveal key regulatory molecules that control effector cascades. Changes in specific phosphorylation index can then be correlated with cellular responses known to be under the control of a particular kinase.²⁴

Although the approach for measuring phosphorylation index reported here is likely to be applicable to a wide spectrum of kinases, it has inherent limitations that require consideration. For example, it is not feasible to use this approach with kinases that require a priming phosphorylation event (i.e. Polo-like Kinase I), since the substrate pool is globally de-phosphorylated by HF treatment. In addition, although we did not detect effects of HF treatment on proteins detectable by silver staining in a whole cell extract, we cannot rule out the possibility that low abundance proteins may be affected. Furthermore, since potential substrates are identified by MS after 2D gel electrophoresis, co-migration of abundant proteins could potentially mask less abundant substrates. Another factor that must be considered is the possibility that kinase inhibitor treatment may result in secondary changes in post-translational protein modification (i.e. ubiquitination) that may preclude phosphorylation, and thereby affect phosphorylation index. In addition, incomplete in-gel substrate refolding may also occur. It is also important to note that substrates that do not respond to inhibitor treatment could potentially represent false positives.

The data reported here underscore the value of systematic analyses of substrate responses to kinase inhibition to inform PD biomarker selection, and the inherent danger of depending on one marker to accurately report on drug efficacy. Furthermore, our studies suggest that the common practice of relying exclusively on phosphospecific anti-substrate antibodies may be less informative, in some cases, than using de-phosphospecific antibodies. In the case of proteins with a high constitutive phosphorylation index, for example, RP2 and Nucleophosmin 1, the fold change in signal observed using phosphospecific antibody upon CX-4945 treatment would be expected to be ~20–25% (Table 1). However the change in de-phosphospecific anti-RP2 antibody signal would be predicted to be greater than 500%.

Although RIKA-based kinase-specific phosphorylation indexing can give in-depth information, this assay is currently low throughput. Further development of the RIKA by integrating high-resolution chromatography and SILAC¹⁴ may enable more comprehensive

kinase substrate profiling, and improve coverage significantly. Wide application of this method in the area of functional phosphoproteomics could greatly facilitate our understanding of the role of protein phosphorylation index in both normal and pathological cell states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Synopsis

Here, we describe a system to globally quantify the phosphorylation index for substrates of a specific kinase by independently quantifying phosphorylated and total substrate molecules in a Reverse In-gel Kinase Assay. Non-phosphorylated substratemolecules are first quantified in the presence and absence of a specific stimulus. Total substrate molecules are then measured after complete chemical de-phosphorylation using hydrogen fluoride treatment, and a ratio of phosphorylated to total substrate is derived.

Figure 1.

Measuring protein phosphorylation index using a 2D RIKA. *In vivo* protein phosphorylation index varies greatly. RIKA “sees” the non-phosphorylated fraction of each substrate (red represents phosphorylated substrate pool, other colors represent non-phosphorylated pool). When kinase activity is inhibited *in vivo*, for substrates that “respond”, the pool of non-phosphorylated protein increases (*cf.* Proteins 1 and 2, panels A and C), and the RIKA autoradiographic signal increases (*cf.* proteins 1 and 2, panels B and D). To measure the total protein pool, complete de-phosphorylation is performed before the RIKA (panels E and F). The ratio of the RIKA signal observed for each protein in the absence (panel B) or presence (panel E) of an inhibitor over the signal after complete de-phosphorylation (panel F) represents the de-phosphorylation index (I^{dp}) $\text{Intensity}^{\text{control}}/\text{intensity}^{\text{total}}$. The phosphorylation index (I^p), can then be calculated as $1 - I^{dp}$. In the examples shown, Protein 2 is the most inhibitor-responsive substrate, and becomes completely hypo-phosphorylated when inhibitor is present ($I^p = 0.80$ vs. $I^p = 0.0$), Protein 1 is less responsive ($I^p = 0.5$ vs. $I^p = 0.38$), while Protein 3 does not respond ($I^p = 1.0$ vs. $I^p = 1.0$).

Figure 2.

Development and validation of a RIKA-based method to measure protein phosphorylation index. A. HF treatment quantitatively de-phosphorylates proteins in a HeLa whole cell protein extract. HeLa cells were cultured in DMEM containing ^{32}P -orthophosphate to metabolically label phospho-proteins. The labeled protein extract was HF-treated, analyzed by SDS-PAGE, and transferred to a PVDF membrane. The membrane was Coomassie blue stained (right panel), and de-phosphorylation efficiency after 1, or 2 hours of HF-treatment or after no treatment (c, control) was measured by autoradiography (left panel). B. HF-treated CK2 substrate can be fully re-phosphorylated. TEBP was *in vitro* phosphorylated to completion by CK2, then de-phosphorylated by HF treatment, and subsequently analyzed in a CK2 RIKA followed by silver staining. M, molecular weight marker. C. Measuring phosphorylation index of standards using RIKA. To generate phosphorylation index standards, TEBP was phosphorylated to completion by CK2 or mock phosphorylated. Phosphorylated and mock-treated TEBP were mixed to create a series of I^p standards using TEBP. As an internal control for protein recovery, a fixed mass of non-phosphorylated ANP32B (another CK2 substrate) was added to each TEBP I^p standard. The mixture was

then analyzed on a CK2 RIKA before, and after HF treatment. The signals were quantified and the I^p was calculated. D. Quantification of data shown in (C). Dark shading represents expected I^p value, light shading represents the experimentally determined I^p value. ANP, ANP32B; M, molecular weight marker; Phos, phosphorylated *in vitro* by CK2.

Figure 3.

Measuring CK2 substrate phosphorylation index in tissue culture cells. HeLa cells were treated with CX-4945 (100 μ M) for 4 hours or mock treated. Whole cell protein extracts were prepared and treated with HF to de-phosphorylate the proteome. 2D CK2 RIKAs were carried out for all 4 lysates. Changes in I^p of CK2 substrates were measured as described above in Figure 2. Black arrows designate GP94, the protein used as an internal control, which is completely non-phosphorylated *in vivo*. a-e, HeLa cell proteins that were hypo-phosphorylated upon CX-4945 treatment: a, Ribosomal Protein P2 (RP2); b, EF-1 β ; c, unidentified human protein; d, Eukaryotic Translation Initiation Factor 5; e, Ras-GTPase-activating Protein SH3-Domain-Binding Protein. B. CK2 substrates respond to CK2 inhibitor CX-4945 treatment in a dose-dependent manner. C. CK2 substrates respond to CK2 inhibitor treatment in a time-dependent manner.

Figure 4.

Validation of change in CK2 substrate phosphorylation index by affinity pull-down. A. Changes in EF-1β phosphorylation index at various CK2 inhibitor CX-4945 concentrations. HeLa cells transfected with a vector expressing His-tagged EF-1β were treated with CX-4945 at various concentrations for 60 minutes beginning 24 hours post-transfection. Tagged EF-1β was purified using nickel resin. Eluates were analyzed by 1D CK2 RIKA and Western blot using an anti-HA antibody. The eluate from a vehicle-treated cell lysate was de-phosphorylated using HF to generate a completely de-phosphorylated standard ($I^{dp} = 1.0$, $I^p = 0.0$). B. Changes in EF-1β phosphorylation index at various CK2 inhibition time points. HeLa cells transfected with a vector expressing His-tagged EF-1β were treated with 10 μM CX-4945 for 10, 30, or 60 minutes beginning 24 hours post-transfection. C. CK2 substrates respond differentially to CX-4945 treatment. Vectors expressing EF-1β and Nucleophosmin were co-transfected into HeLa cells. Cells were treated with CX-4945 (100 μM) for 60 minutes beginning 24 hours post-transfection. Responses of EF-1β and Nucleophosmin to the inhibitor treatment were determined by measuring their phosphorylation indexes before and after treatment. D. The CK2 substrate PDIA6 was expressed in HeLa cells as a His+HA tagged protein. Cells were treated with 100 μM CX-4945 24 hours after transfection, or mock treated, for 60 minutes before harvesting. Purified PDIA6 from mock treated cells was de-phosphorylated with HF.

Figure 5.

Measuring CK2 inhibitor CX-4945 efficacy in mice. CX-4945 (100 mg/kg) was administered IV to adult FVB/N mice and PBMCs were harvested after 30 minutes. Changes in I^p of CK2 substrates were measured as described above in Figure 2. Black arrows designate GP94, the protein used as an internal control, which is completely non-phosphorylated *in vivo*. Circles, CK2 substrates that did not respond to inhibitor treatment. f–j, mouse PBMC proteins that were hypo-phosphorylated upon CX-4945 treatment; f, unidentified mouse protein; g, Rp2; h, Ef-1 β ; i, Purine rich protein b (Purb); j, Suppression of tumorigenicity 13.

Figure 6.

Kinetics of CX-4945 responsiveness for selected CK2 substrates. A. PBMC whole-cell protein lysates from mice treated with CX-4945 for various periods were analyzed in 2D CK2 RIKAs. The signal intensities of four substrates were quantified for all time points. For each time point, the highest signal intensity among the time points was defined as maximum response ($R_{\max}$). The ratio of the signal intensity at each time point after inhibitor treatment over $R_{\max}$ is defined as the response index (I^R). B. Response index for mouse Rp2, Ef-1 β , Purb, and Suppression of tumorigenicity 13 are plotted against CX-4945 exposure time.

CX-4945-induced change in I^p and I^{dp} in HeLa cells and mouse PBMCs. The I^p , I^{dp} , and fold change in both indexes for CX-4945-responsive proteins shown in Figures 3 and 5 were calculated.

Table 1

CX-4945 induced I^{dp} and I^p changes in HeLa cells					
Protein Spot	Control I^{dp}	Inhibitor I^{dp}	Control I^p	Inhibitor I^p	Fold Change I^p
a	0.02	0.96	0.98	0.04	45.38
b	0.02	0.72	0.98	0.28	46.02
c	0.15	0.50	0.85	0.50	3.41
d	0.10	0.44	0.90	0.56	4.49
e	0.11	0.40	0.89	0.60	3.73
CX-4945 induced I^{dp} and I^p changes in mouse PBMC					
f	0.03	0.18	0.97	0.82	6.01
g	0.03	0.67	0.97	0.33	21.36
h	0.04	0.13	0.96	0.87	3.00
i	0.06	0.44	0.94	0.56	6.85
j	0.04	1.00	0.96	0.00	28.81
					∞