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Proteome-Wide Characterization of Phosphorylation-Induced Conformational Changes in Breast Cancer

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Abstract

Because of the close link between protein function and protein folding stability, knowledge about phosphorylation-induced protein folding stability changes can lead to a better understanding of the functional effects of protein phosphorylation. Here, the Stability of Proteins from Rates of OXidation (SPROX) and Limited Proteolysis (LiP) techniques are used to compare the conformational properties of proteins in two MCF-7 cell lysates, including one that was and one that was not dephosphorylated with alkaline phosphatase. A total of 168 and 251 protein hits were identified with dephosphorylation-induced stability changes using the SPROX and LiP techniques, respectively. Many protein hits are previously known to be differentially phosphorylated and/or differentially stabilized in different human breast cancer subtypes, suggesting that the phosphorylation-induced stability changes detected in this work are disease related. The SPROX hits were enriched in proteins with aminoacyl-tRNA ligase activity. These enriched protein hits included many aminoacyl-tRNA synthetases (aaRSs), which are known from previous studies to have their catalytic activity modulated by phosphorylation. The SPROX results revealed that the magnitudes of the destabilizing effects of dephosphorylation on the different aaRSs were directly correlated with their previously reported aminoacylation activity change upon dephosphorylation. This substantiates the close link between protein folding and function.

Graphical Abstract

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Supplementary Text: Additional information is provided about the **Materials and Methods** including more details about the SILAC-SPROX data analysis.

SUPPORTING INFORMATION. The following files are available free of charge.

INTRODUCTION

Reversible phosphorylation is one of the most prevalent post-translational modifications (PTM) in cellular metabolism. It is also an important PTM in cellular signal transduction, and it plays a role in nearly all cellular processes by regulating protein functions. This control of protein function is accomplished by modulating protein-protein interactions and protein conformations, and it typically involves altering a protein's biophysical properties, such as its folding stability and kinetics.¹⁻³ Using modern MS-based, phospho-proteomic techniques, a variety of biological samples (e.g. cell lines, biological fluids, and tissues) can be qualitatively and quantitatively analyzed for the presence of phosphorylated proteins.^{2, 4-8} Phospho-proteomic studies on a wide variety of biological samples have served to catalogue a number of different phosphorylation sites in proteins. However, one challenge in phospho-proteomic studies is identifying the functional significance of protein phosphorylation events.

The first functional studies of protein phosphorylation were reported as early as the 1950s.⁹⁻¹⁰ In the intervening 60 years, the structural and biophysical consequences of protein phosphorylation have been studied using various techniques including X-ray diffraction, autoradiography, circular dichroism (CD), and nuclear magnetic resonance (NMR).¹¹⁻¹⁶ Studies have shown that protein phosphorylation can impact protein structure in many different ways including: stabilizing specific conformations, effecting allosteric conformational changes, acting as a steric blocking agent, or promoting protein order/disorder transitions.^{3, 17} Thus, there is much evidence that phosphorylation events can induce protein conformational changes. However, the techniques traditionally used to decipher such changes require relatively high concentrations of purified proteins. Therefore, they are not amenable to high-throughput and large-scale analyses. Protein phosphorylation events can also modulate protein-protein and protein ligand interactions. It has been estimated that about one third of the protein complexes in the human database have significant binding energy changes upon phosphorylation.¹⁸ Unfortunately, methods for the large-scale and unbiased search for proteins with such phosphorylation-induced functions (i.e., the ability to globally assess the general effects of phosphorylation on protein conformation and ligand binding) are lacking.

Recently, several proteomic techniques have been developed for the large-scale characterization of protein conformations, thermodynamic stabilities, and protein-ligand interactions.¹⁹⁻²⁸ Included in these techniques are the stability of proteins from rate of oxidation (SPROX) and limited proteolysis (LiP) techniques, which both enable the study of protein conformational changes in complex protein mixtures. In SPROX, the chemical denaturant dependence of a hydrogen peroxide-mediated oxidation reaction involving methionine side chains is used to generate information about the global thermodynamic stability of proteins. In LiP, a limited proteolysis reaction involving a non-specific protease and the native states of proteins is used to generate information about the more local conformational properties of proteins. Both the SPROX and LiP techniques have been used to compare the stabilities and structural changes of proteins in different biological states^{22, 24, 29} as well as to identify the protein targets of ligand.^{27, 30}

Described here is an application of the SPROX and LiP techniques to identify phosphorylation-induced conformational changes in proteins derived from a cell culture model of breast cancer, the estrogen receptor positive MCF-7 breast cancer cell line. A number of phosphoproteomic studies have been performed on the proteins from this and other cell culture models of breast cancer. However, these studies have primarily focused on mapping the specific sites of protein phosphorylation. Here, the SPROX and LiP techniques are utilized to study the protein folding stability changes, associated with protein phosphorylation on the proteomic scale. Because of the close relationship between stability and function, the described approach provides a means by which to generally probe the functional effects of phosphorylation including phosphorylation-induced conformational and/or ligand binding changes.

EXPERIMENTAL

Cell Culture and Preparation of Cell Lysates

The human breast cancer cell line, MCF-7 from ATCC, was cultured in a humidified 37 °C incubator with 5% CO₂. Light SILAC-labeled MCF-7 cells were cultured following the ATCC guidelines. Heavy SILAC-labeled cells were cultured using heavy labeled lysine and arginine according to standard protocols.³¹ The heavy labeled lysine was enriched with six ¹³C and two ¹⁵N (Cambridge Isotope Laboratories, Inc.). The heavy labeled arginine was enriched with six ¹³C.

MCF-7 cells were lysed by mechanical disruption using a disruptor genie (Scientific Industries) and 1 mm diameter zirconia/silica beads (Biospec) in 20 mM Tris-HCl buffer (pH 9.0) containing 10 mM NaCl, 1 mM MgCl₂ and protease inhibitor cocktail (1 mM AEBSF, 500 μ M Bestatin, 15 μ M E-64, 20 μ M Leupeptin, and 10 μ M Pepstatin A (Thermo Pierce)). The cell lysates were centrifuged at 14000 $\times$ g for 15 min at 4 °C, and the supernatant was used to generate (+) and (–) phosphatase samples. The light and heavy labeled lysates were normalized to same total protein concentration (4–5 mg/ml) using lysis buffer.

Alkaline phosphatase from bovine (Sigma-Aldrich) was added to the heavy labelled lysate at a ratio of 0.5 units/ μ g total protein and incubated at 37 °C for 16 h to generate the (+) phosphatase sample. The light labeled cell lysate was subjected to the same treatment except no alkaline phosphatase added.

SILAC-LiP Analyses

The (+) and (–) phosphatase lysates were adjusted to 2 mg/ml with 20 mM HEPES (pH 7.5), 150 mM KCl and 10 mM MgCl₂²². The (+) and (–) phosphatase lysates were each divided into two 100 μ L aliquots (four equal aliquots total) to generate a double digestion (DD) group and single digestion (SD) group. Proteinase K from Tritirachium album (Sigma) was added to the two samples in the DD group, which included a (+) and a (–) phosphatase sample. The final ratio of Proteinase K to total protein was 1:100 (w/w) enzyme. The mixtures were incubated for 5 min at room temperature and the digestion reaction were quenched upon addition guanidine hydrochloride (Sigma) to a final concentration of 7.4 M.

The digestions were heated at 100 °C for 3 min to ensure complete denaturation. The SD group was treated according to the same procedure as the DD group, except no proteinase K added. The light and heavy samples in the DD group were combined, as were the light and heavy samples in the SD group. The resulting light and heavy sample mixtures were combined and prepared for bottom-up proteomics analysis using trypsin (see below).

SILAC-SPROX Analyses

The SILAC-SPROX protocol employed here was similar to that which has been previously described.²⁶ Briefly, 20 μL of the (+) and (–) phosphatase lysates were diluted into 75 μL buffer solutions containing of 20 mM phosphate (pH 7.4) and urea concentrations ranging from 0 to 10 M. The final concentration of urea in each set of denaturant-containing buffers ranged from 0 to 8 M. The (+) and (–) samples were equilibrated in each urea buffer for 2 h. The methionine oxidation reaction was initiated by adding 5 μL hydrogen peroxide (Sigma-Aldrich) into each buffer to achieve a final concentration of 0.5 M. The reactions in the denaturant-containing buffers were allowed to proceed for 6 min, before they were quenched with 653 μL of a 375 mM L-methionine (Sigma-Aldrich) solution. The (+) and (–) phosphatase samples at each concentration of urea were combined. Trichloroacetic acid (TCA) (Sigma-Aldrich) was added at final concentration of 16% (w/v) to precipitate the protein. After incubation on ice for 16–18 h, the samples were centrifuged at $8000 \times g$ and 4 °C for 30 min. Supernatants were discarded and the protein pellets were rinsed with 300 μL ice-cold ethanol three times. The pellets were dried under vacuum and re-dissolved in 60 μL of 0.5 M triethylammonium bicarbonate (TEAB) (Sigma-Aldrich) containing 3 μL of a 2% stock solution of sodium dodecyl sulfate (SDS). The protein solutions were then submitted to standard bottom-up proteomics analyses.

Bottom-Up Proteomics Sample Preparation

The protein samples generated in the SILAC-LiP and SILAC-SPROX experiments were treated with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (ThermoFisher) (5 mM) for 1 h at 60 °C. The proteins were reacted with 10 mM methylmethane thiosulfonate (MMTS) (Sigma-Aldrich) at room temperature for 15 min to block cysteine side chains. The SILAC-LiP samples were diluted with 0.5 M triethylammonium bicarbonate (TEAB) buffer (Sigma) such that the final concentration of GdmCl was less than 2 M. Trypsin was added to each of the SILAC-LiP and SILAC-SPROX samples. In all cases, the ratio of trypsin to total protein was 1:20 to 1:50. The trypsin digestions were allowed to proceed at 37 °C for 16–18 h before quenching with 10 % trifluoroacetic acid (TFA) to pH 2–3. The digested samples were desalted using C18 resin (The Nest Group) according to the manufacturer's protocol.

LC-MS/MS Analyses

The desalted samples were analyzed on a Q-Exactive Plus mass spectrometer system (Thermo Scientific, Inc.) equipped with a nanoAcquity UPLC system (Waters Corp.) and a nano-electrospray ionization source. Samples were trapped on a Symmetry C18 300 mm $\times$ 180 μm trapping column for 3 min at 5 $\mu\text{L}/\text{min}$ (99.9/0.1 v/v water/acetonitrile 0.1% formic acid), and separated on a 75 μm $\times$ 250 mm column packed with 1.7 μm Acquity HSST3 C18 stationary phase (Waters Corp.). Peptides were separated using a gradient of 3 to 30% acetonitrile with 0.1% formic acid over 90 min at a flow rate of 0.4 $\mu\text{L}/\text{min}$. Data collection

was performed in a data-dependent acquisition (DDA) mode with a resolution of 70,000 (at m/z 200) for full MS scan from m/z 375–1600 with a target AGC value of 1×10^6 ions, followed by 20 product ion scans at a resolution of 17,500 (at m/z 200), using an AGC target value of 1×10^5 ions, a max fill time of 60 ms, and normalized collision energy of 30 V.

The three biological replicates of the double and single digestion samples generated in the SILAC-LiP experiments were each analyzed by LC-MS/MS in triplicate. The twelve SILAC-SPROX protein samples generated in each of the three biological replicates were subject to a single LC-MS/MS run (i.e., each biological replicate included twelve LC-MS/MS runs).

The raw LC-MS/MS data were searched by MaxQuant 1.5.2.8 against the 20265 human proteins in the 2014-04 release of the UniProt Knowledgebase and exported into excel spreadsheet. In the SILAC-LiP experiments, searches were performed using trypsin as the enzyme with semi-specificity. In the SILAC-SPROX experiments, searches were performed using trypsin as the enzyme with specific cleavages after K and R. All searches set SILAC labeling of lysine and arginine, and MMTS as fixed modifications. Oxidized methionine, glutamine and asparagine deamination, and acetylation of the protein N-terminus were set as variable modification. The mass tolerances for precursor ions was set to 20 ppm and 10 ppm for first and main search respectively. The mass tolerance for fragment ions was set as 0.02 Da. Match between runs and re-quantification were used for each search. The remaining parameters were set as the default values in MaxQuant. Peptides with false discovery rates <1% and positive heavy to light (H/L) ratios were used for subsequent data analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange³² Consortium via the PRIDE³³ partner repository with the dataset identifiers PXD007878 and PXD007916 for the SILAC-LiP and SILAC-SPROX data, respectively.

Data Analyses

In the SILAC-LiP experiment, the data were treated as described previously.²⁹ Briefly, in each biological replicate, a median H/L ratio was determined for each protein using all the peptides identified from the protein in the single digestion group. The H/L ratios of peptides from the same protein in both single and double digestion groups were then normalized by the protein median in each biological replicate. This normalization accounted for protein abundance differences. The normalized H/L ratios were transformed to log base 2 values. Only peptides identified two or more times in both single and double digestion groups were considered for hit selection, which involved the use of a Student's two-tailed t-test to identify statistically significant H/L ratio differences between the single and double digestion groups. Peptides with p-value less than 0.05 were selected as hits. Excel spreadsheets containing all the peptides and proteins identified in the three biological replicates of the SILAC-LiP experiment as well as the peptides and proteins assayed and selected as hits in the t-test are summarized in Table S-1 in the Supporting Information.

In the SILAC-SPROX experiment, the data were analyzed as previously described.³⁴ Briefly, a Mathematica script, developed in house, was used to process the data and to determine final hits. The script imported the output files from MaxQuant, and normalized the H/L ratio of methionine containing peptide with the H/L ratio of non-methionine peptide from the

same protein. Only peptides identified in at least 7 different denaturant concentrations were assayed. The peptide hit selection procedure, which was identical to that previously described for SILAC-SPROX data,³⁴ involved the identification of peptides with a significant fold-change (i.e., in the outlying 5th and 95th percentiles of the H/L ratios of the non-methionine-containing peptides) at two or more consecutive denaturant concentrations. The H/L ratio vs. [Urea] data of the hits also had to be well-fit to the equations (see Supporting Information) we previously developed to describe SILAC-SPROX data.³⁴ The peptides and proteins assayed and identified as hits in the SILAC-SPROX experiments are summarized in Table S-2 in the Supporting Information.

RESULTS AND DISCUSSION

SILAC-LiP Analysis

Prior to the SILAC-LiP analysis, heavy and light labeled MCF-7 cell lysates were treated with and without phosphatase, respectively. The cell lysate samples were then subjected to a SILAC-LiP analysis (Figure 1), which was essentially identical to that previously described in reference 29. In total over 6,000 peptides from ~1300 proteins were identified in the LC-MS/MS analyses of the double and single digestions samples in the 3 biological replicates. Ultimately, 3490 peptides from ~700 proteins were assayed for proteinase K susceptibility changes induced by dephosphorylation. The assayed peptides included those that were successfully identified and quantified in two or more LC-MS/MS runs of the double and single digestion groups. From these assayed proteins, 566 unique peptides from 251 proteins were assigned as hits with statistically significant H/L ratio value differences ($p < 0.05$) (Table S-1).

In order to help eliminate potential false positives, the 566 peptide hits were compared to the peptide hits previously reported in a control experiment in which the SILAC-LiP protocol was used to analyze the proteins in light- and heavy-labelled MCF-7 cell lysates.²⁹ This comparison identified 31 peptide hits that were also identified as hits in the MCF-7 control experiment (i.e., they appeared as hits even when the heavy cell lysate was not treated with alkaline phosphatase). These were removed from the hit lists as they were likely false positives. An average fold-change value was calculated for each peptide and plotted versus the p-value of the peptide (Figure 2). Most hits (~90%) were found to have fold-changes in the range of 1.1 to 2.3, with the median value being 1.3. These characteristics of the hit peptides are similar to those observed for the hits in an earlier SILAC-LiP study on the proteins in several different cancer cell lines.²⁹

Among the 535 peptide hits, ~15% were semi-tryptic peptides resulting from proteinase K cleavage. This is consistent with semi-tryptic peptides being more difficult to detect and quantify compared to fully tryptic peptides. It is also similar to that observed in a previously reported SILAC-LiP experiment.²⁹ We note that the semi-tryptic peptide hits in the SILAC-LiP experiment are not due to non-specific trypsin cleavages as any semi-tryptic peptides generated from the trypsin digestion would be present in both the L and H samples and have a L/H ratio of 1. Of the 535 peptide hits, 57% were found to be more susceptible to proteinase K cleavage upon treatment with phosphatase, with rest being less susceptible to proteinase K. A large fraction (84%) of the 535 peptide hits in the SILAC-LiP experiment

were identified in both the single and double digestion groups in two or more independent biological replicates. Among the 245 protein hits, 38% were identified with two or more peptide hits.

A search of the PhosphoSitePlus³⁵ Knowledge database (<http://www.phosphosite.org>) revealed that 157 of the 245 protein hits have one or more previously reported phosphorylation site(s). The experimental approach used here was designed to detect the overall influence of dephosphorylation on unpurified proteins in a complex cell lysate. Therefore, it is not surprising that a fraction of the hit proteins did not have known phosphorylation sites. Presumably these proteins hits are the result of indirect effects from altered protein-protein interactions involving a phosphorylated protein (e.g., dephosphorylation of a site on one protein either induces or precludes the binding of a second protein). We note that a small fraction (7%) of the SILAC-LiP peptide hits were actually known to contain phosphorylation sites in their sequence.

SILAC-SPROX Analysis

The heavy and light labeled MCF-7 cell lysate samples treated with and without phosphatase, respectively, were also subject to the SILAC-SPROX protocol outlined in Figure 3. The proteomic coverage observed in the SILAC-SPROX experiment are summarized in Table 1. Overall, 2939 peptides from 1023 proteins were assayed, and 212 peptides from 168 proteins showed dephosphorylation-induced thermodynamic stability changes (Table S-2). Representative SILAC-SPROX data sets for several hit peptides are shown in Figure 4. The dephosphorylated samples contained alkaline phosphatase. Thus, it is possible that some of the thermodynamic stability changes observed in the SILAC-SPROX (and SILAC-LiPs) protein hits are due to alkaline phosphatase interactions. However, the alkaline phosphatase concentration (1 μ M) was relatively low; therefore, to appear as a hit the alkaline phosphatase binding interaction would have to be relatively tight (e.g., K_d in the nanomolar range). While such alkaline phosphatase binding may explain a small fraction of hits, it is unlikely to explain the relatively large number of detected hits or generate the correlations of conformational changes with changes in protein function that are described below.

A search on the PhosphoSitePlus³⁵ database revealed that 130 of the 168 proteins in the final hit list have at least one previously reported phosphorylation site. The dephosphorylation induced stability changes detected in these 130 proteins are likely to be directly related to the phosphorylation. The stability changes detected in the remaining 38 protein hits are likely due to indirect effects of protein phosphorylation as described above. We note that a small fraction (5%) of the SILAC-SPROX peptide hits were actually known to contain phosphorylation sites in their sequence.

Approximately half of the peptide hits resulted from dephosphorylated-induced stabilizations. Ultimately, 88 peptides had sufficient data to quantitatively evaluate the transition midpoint and folding free energy changes (i.e., $C_{1/2}$ and G_f values, respectively). The median $C_{1/2}$ and G_f values were 2.7 mol·L⁻¹ and 1.6 kcal·mol⁻¹, respectively. Over 80% of the hit peptides had $C_{1/2}$ values ranging from -5.0 to 5.0 mol·L⁻¹ and G_f values ranging from -5.0 to 5.0 kcal·mol⁻¹. However, it is important to note

that the measured $C_{1/2}$ and G_f values may not necessarily be the results of a single site in the protein being dephosphorylated but rather the aggregate effect of all the dephosphorylations on the protein.

An overrepresentation analysis of the SILAC-SPROX hit proteins compared to the assayed protein using GOrilla³⁶ revealed a statistically significant enrichment of proteins with aminoacyl-tRNA ligase activity (GO:0004812; p-value = 0.0005) including a number of aminoacyl-tRNA synthetases (aaRSs). aaRSs are essential enzymes in the production of proteins in the cell, where they catalyze the transfer of AMP activated amino acid to 3' end of their cognate tRNA to form aa-tRNA. Several studies have demonstrated that phosphorylation events play a key role in regulating the biological activity of aaRSs.^{37–39} For example, the phosphorylation of Ser886 and Ser999 in glutamyl-prolyl tRNA synthetase (EPRS) has been reported to be essential to the function of this aaRS.³⁸

A total of 37 protein hits were identified in both the SILAC-SPROX and SILAC-LiP experiments. These 37 proteins represent only a small percentage of the SILAC-SPROX and SILAC-LiP hits (15% and 22%, respectively). The relatively low fraction of overlapping protein hits is not surprising, as the LiP and SPROX experiments probe different types of protein conformational changes. The LiP experiment is amenable to the detection of more local conformational changes (e.g., the unravelling an α -helix); whereas the SPROX experiment is amenable to the detection of more global conformational changes (e.g, a change in the opening and closing rates of a protein folding domain). A similarly low percentage of overlapped hits (~20%) observed between LiP and SPROX experiments were also observed in a disease state studies on human breast cancer cell lines.²⁹

Comparison with other Large-Scale Phospho-Proteomic Studies

In order to better understand the disease relevance of the stability changes observed in this work we correlated the SILAC-SPROX and LiP protein hits with traditional quantitative phospho-proteomic results reported on six cell culture models of human breast cancer including: MCF-7, HCC1937, BT549, BT20, MDA-MB-231, and MDA-MB-468⁴⁰ cells that differ in molecular features and degree of invasiveness. The phospho-proteomic results from the MCF-7, HCC1937, BT549, BT20, MDA-MB-231, and MDA-MB-468 cell lines included 13730 phosphorylated sites in 3880 proteins in total. The conformational studies in this work involved phosphatase treated proteins from an MCF-7 cell lysate. Therefore, the proteins with sites that were phosphorylated in MCF-7 and not phosphorylated (or less so), in the above cell lines were of most interest. Summarized in Table 2 are the numbers of proteins with such differential phosphorylation in the above cell lines and those that overlapped with the protein hits identified in the SPROX and LiPs experiments described in this work. Because changes in conformation are closely linked to changes in protein function, the 49 overlapping protein hits that were found (see Table S-1 and S-2 in the Supporting Information) are likely to be functionally regulated by phosphorylation in the different breast cancer phenotypes. Indeed, some of these 49 proteins (e.g., PGAM1⁴¹, STUB1⁴², EBP1⁴³, TACSTD2⁴⁴, and TPD52L2⁴⁵) are known to have cancer related functions induced by phosphorylation.

We have previously used SILAC-SPROX to detect breast cancer related conformational changes in proteins derived from selected cell culture models of breast cancer.^{24, 34} In these earlier studies, the molecular basis of the detected conformational changes was not clear. As part of this work, we examined the correlation between the dephosphorylation-induced changes observed in the MCF-7 cell line used in this work and the general breast cancer related conformational changes observed in our earlier studies.^{24, 34} We hypothesized that some of the protein stability differences observed in our earlier studies, which compared the thermodynamic stability of proteins in different cell culture models of breast cancer to those in the MCF-7 cell line (see Table 3), might be the result of differential phosphorylation in the different breast cancer cell lines. Therefore, we compared the SILAC-SPROX protein hits generated in this work to the proteins hits found in our earlier SILAC-SPROX studies, and ultimately searched for the overlapping proteins in the available phospho-proteomic data summarized in Table 2. Out of the 11 and 90 overlapped proteins that have different stability in the MDA-MB-231 or MDA-MB-468 cell line compared to MCF-7 (see Table 3), we found 3 and 9 of these protein hits, respectively, that were less phosphorylated in MDA-MB-231 and MDA-MB-468 (respectively) than in MCF-7 (see Table S-2E in Supporting Information). This correlation of the SILAC-SPROX results in the current dephosphorylation study with the results in our earlier SILAC-SPROX on the different cancer cell lines,^{24, 34} suggests that the stability changes detected for these 3 and 9 proteins are due to dephosphorylation-induced changes in those cell lines.

Correlation of Conformational Changes with Changes in Protein Function

Some protein hits in this study have previously reported functional changes induced by phosphorylation. For example, pyruvate kinase (PKM2) has been reported to have reduced activity by the direct phosphorylation on Y105, which disrupts formation of active tetrameric PKM2 by releasing cofactor fructose-1,6-bisphosphate (FBP).⁴⁶ Our LiP results suggest that after dephosphorylation, PKM2 has a more compact conformation around the FBP binding pocket and a more protease susceptible conformation in the vicinity of Y105 (Figure 5a). Our SILAC-SPROX results also revealed a dephosphorylation-induced stabilization for a methionine-containing peptide in the FBP binding pocket of PKM2. The LiP and SPROX data are consistent with the previous study on the functional effect of Y105 phosphorylation, as higher affinity to FBP caused by dephosphorylation of Y105 would probably stabilize the binding domain of PKM2.

Another protein hit, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is also well known to have its function regulated by post-translational modifications.^{47–48, 49–52} In our study, multiple peptides from GAPDH showed significant protease susceptibility changes upon dephosphorylation. These peptides surround the NAD binding pocket of the protein (Figure 5b). The SPROX and LiP data collected here suggests that dephosphorylation of amino acid residues around the GAPDH binding pocket destabilizes this region of the protein. This is consistent with the results of earlier studies, where it was suggested that phosphorylation of Y94, S98 and T99 in GAPDH increased the protein's NAD binding affinity.⁵³

The SILAC-SPROX experiment enabled us to quantify the protein stability changes induced by dephosphorylation for a number of aaRSs (Table S-3). Most of the aaRSs were destabilized upon alkaline phosphatase treatment, indicating that phosphorylation of aaRSs stabilizes these proteins. We also found that the magnitudes of the destabilizing effects of aaRS dephosphorylation were directly correlated with their previously reported aminoacylation activity change upon dephosphorylation (with only one exception, histidyl-tRNA synthetase) (Figure 6).⁵⁴ This correlation helps substantiate the close connection between protein stability and protein function.

CONCLUSIONS

A large-scale proteomic analysis of the protein conformational changes associated with phosphorylation in MCF-7 breast cancer cells using the SRPOX and LiP techniques resulted in the identification of a subset of 168 and 251 proteins, respectively, with phosphorylation-induced stability changes. Many of the protein hits are known to be differentially phosphorylated and/or differentially stabilized in different human breast cancer subtypes, suggesting that the observed phosphorylation-induced stability changes detected in this work are disease related. A bioinformatics analysis revealed that the SPROX hits were enriched in proteins with aminoacyl-tRNA ligase activity, including many aaRSs that are known to have their catalytic activity modulated by phosphorylation events. The SPROX results, which enabled the magnitudes of the stabilizing effects of dephosphorylation on the different aaRSs to be evaluated, revealed that the magnitudes of the destabilizations were directly correlated with their previously reported change in aminoacylation activity upon dephosphorylation. This substantiates the close link between protein folding and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic presentation of the SILAC-LiP protocol used in this work. (A) Experimental workflow for generation of the single and double digestion samples. (B) Data expected from single and double digestion samples.

Figure 2.

Volcano plot showing the statistical significance of the H/L ratios for the peptides assayed in the SILAC-LiP study. Gray points represent the 2924 non-hit peptides ($p\text{-value} > 0.05$). Red points represent the 475 hit peptides ($p\text{-value} < 0.05$) that were identified in two or more biological replicates in both single and double digestion groups. Blue points represent the 91 hit peptides that were identified in only one biological replicate in at least one of the single or double digestion groups.

Figure 3.
Schematic presentation of SILAC-SPROX protocol use in this work.

Figure 4.

Representative SILAC-SPROX data. (a) Data obtained on the oxidized methionine-containing peptide VCM(ox)DFNIIR from isoleucine--tRNA ligase, which was destabilized upon dephosphorylation. (b) Data obtained on the methionine-containing peptide LTGMAFR from GAPDH, which was also destabilized upon dephosphorylation. (c) Data obtained on the oxidized methionine-containing peptide (ac)M(ox)EVTGDAGVPESGEIR from the DNA fragmentation factor subunit alpha, which was stabilized upon dephosphorylation. In all plots, the data point not included in each fitting is indicated with a red X. The solid black curves represent the best fit of the data to equation 1 (a) and (c) or 2 (b) in the Supporting Information. The blue and red dotted curves represent the predicted SPROX curves (i.e., peptide intensity versus [urea] data using arbitrary units for better visualization) for the heavy and light labeled peptides. The dashed vertical lines indicate the $C_{1/2,SPROX}$ values determined from the SPROX curves.

Figure 5.

Schematic representation of the three-dimensional structure of two selected protein hits identified in both the LiP and SPROX experiments. (a) Three-dimensional structure of PKM2 in complex with FBP, K⁺, Mg²⁺ and oxalate ion (PDB:3BJF). Y105 was shown in green. The regions to which selected peptide hits identified in SPROX mapped are shown in yellow, and both were stabilized in the dephosphorylated sample. (b) Three-dimensional structure of GAPDH in complex with NAD (PDB:1ZNQ). Y94, S98 and T99 were shown in green. The region to which selected the peptide hit identified in SPROX mapped is shown in yellow, and it was destabilized in the dephosphorylated sample. In both (a) and (b) the regions to which selected peptide hits identified in LiPs mapped are shown in red and blue, depending on whether the hit peptide was more or less (respectively) susceptible to proteolysis. Images were generated in Kinemage, Next Generation (KiNG).⁵⁵

Figure 6.

Correlation between the dephosphorylation-induced changes in folding free energies ($\Delta\Delta G_f$) measured for aaRSs hits identified in SILAC-SPROX experiment and the dephosphorylation-induced changes in activity of the aaRSs determined elsewhere.⁵⁴ The filled circles represent aaRSs with defined G_f values and were included in the linear regression analysis. The open circles represent aaRSs that were not included in the linear regression analysis because either (1) the SILAC-SPROX data points were too few to calculate a G_f value, which is the case for arginine (Arg) tRNA-synthase, or (2) the data point is an outlier, which is the case for histidine (His) tRNA-synthase data point that significantly reduced the correlation coefficient ($R^2 = 0.2238$). The G_f and activity data used in the correlation are summarized in Table S-3 in the Supporting Information.

Table 1

Proteomic coverages obtained in SILAC-SPROX experiments on proteins in the MCF-7 cell lysate.

Biological Replicate	Identified Met-Containing Peptides (Proteins)	Assayed Met-Containing Peptides (Proteins) ^a	Hit Peptides (Proteins)
1	3171 (924)	985 (461)	212 (168)
2	5300 (1154)	2437 (833)	
3	5454 (1130)	2337 (800)	
Total	7024 (1538)	2939 (1023)	

^a Assayed peptides include those identified and quantified in samples from at least 7 different urea concentrations.

Table 2

Summary of proteins with lower levels of phosphorylation in five human breast cancer cell lines compared to the MCF-7 cell line.

Cell Line	Less Phosphorylated Proteins ^a	Proteins Assayed in LiP / SPROX ^b	Protein Hits in LiP / SPROX ^c
HCC1937	1392	33 / 63	21 / 12
BT549	1138	33 / 59	21 / 15
BT20	1120	33 / 63	21 / 12
MDA-MB-231	1140	31 / 55	21 / 16
MDA-MB-468	1124	32 / 65	22 / 11
Total	2048	54 / 100	34 / 24

^aProteins with one or more site(s) that are less phosphorylated in five human breast cancer cell lines than in the MCF-7 cell line as determined from the data in reference 40.

^bProteins assayed by LiP / SPROX in this work that have one or more site(s) less phosphorylated in five human breast cancer cell lines than in MCF-7.

^cProteins hits discovered by LiP / SPROX in this work that have one or more site(s) less phosphorylated in five human breast cancer cell lines than in MCF-7.

Table 3

Protein hits identified in SPROX with previously reported stability differences in four human breast cell lines as compared to proteins in the MCF-7 cell line.

Cell Line	Proteins with Previously Reported Stability Changes	Proteins Assayed in SPROX ^c	Protein Hits Identified in SPROX ^d
MCF-10A	84 ^a	36	9
MDA-MB-231	102 ^a	54	11
MDA-MB-468	445 ^b	367	90
BT-474	242 ^b	186	53
Total	645	449	98

^aFrom reference 24.

^bFrom reference 34.

^cProteins assayed in the SPROX experiments described in this work that have reported stability changes in the four human breast cell lines as compared to the proteins in the MCF-7 cell line.

^dProteins hits discovered in using SPROX in this work that have reported stability changes in the four human breast cell lines as compared to the proteins in the MCF-7 cell line.