A Multidimensional Chromatography Technology for In-depth Phosphoproteome Analysis*[S]

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Protein phosphorylation is a post-translational modification widely used to regulate cellular responses. Recent studies showed that global phosphorylation analysis could be used to study signaling pathways and to identify targets of protein kinases in cells. A key objective of global phosphorylation analysis is to obtain an in-depth mapping of low abundance protein phosphorylation in cells; this necessitates the use of suitable separation techniques because of the complexity of the phosphoproteome. Here we developed a multidimensional chromatography technology, combining IMAC, hydrophilic interaction chromatography, and reverse phase LC, for phosphopeptide purification and fractionation. Its application to the yeast *Saccharomyces cerevisiae* after DNA damage led to the identification of 8764 unique phosphopeptides from 2278 phosphoproteins using tandem MS. Analysis of two low abundance proteins, Rad9 and Mrc1, revealed that ~50% of their phosphorylation was identified via this global phosphorylation analysis. Thus, this technology is suited for in-depth phosphoproteome studies. ***Molecular & Cellular Proteomics 7:1389–1396, 2008.***

Cells are highly responsive to their environment. Protein phosphorylation is a widely used post-translational modification that regulates many biological processes in cells. The phosphoproteome, referring to the phosphorylation profile of cells, undergoes many changes in response to various stimuli. Recent advances in MS have made it possible to identify thousands of phosphopeptides (1–5). Furthermore various stable isotope labeling methods have been used to quantify changes of protein phosphorylation in cells (6–8). These studies illustrate the potential of phosphoproteomics technology to study phosphorylation-mediated signal transduction processes on a large scale.

The phosphoproteome in cells is highly complex, containing phosphorylation of both high and low abundance proteins on multiple sites. To identify phosphorylation of low abundance proteins in a global phosphorylation analysis, it is necessary to have an in-depth mapping of the phosphoproteome. Toward this goal, several methods were developed to purify phosphopeptides prior to their MS analysis, including the use of titanium dioxide, IMAC, and others (1, 9–11). These methods allowed a high degree enrichment of phosphopeptides for subsequent analysis. To analyze the purified phosphopeptides, the use of reverse phase-based HPLC (RP-HPLC)[1] and MS/MS can identify hundreds of phosphopeptides in a single analysis; however, this alone is insufficient for an in-depth mapping of the phosphoproteome.

Strong cation exchange (SCX) chromatography is commonly used to fractionate peptides (12). Recently an SCX column was used to fractionate phosphopeptides, and as a result, many more phosphopeptides were identified (2, 3, 6, 8). Clearly fractionation of phosphopeptides is necessary for a higher coverage of the phosphoproteome. However, it was found that some phosphopeptides did not bind to the SCX column, and other phosphopeptides were mostly eluted from the SCX column earlier than unphosphorylated peptides (13).[2] These observations indicated a relatively weak binding between the SCX resins and phosphopeptides. To analyze phosphopeptides using MS, RP-HPLC is commonly used to further fractionate the phosphopeptides. It was observed that the presence of salt, regardless whether it is volatile or not, would compromise the binding and separation of many phosphopeptides via RP-HPLC. This is likely because many phosphopeptides are hydrophilic and bind less tightly to RP-HPLC columns. Unfortunately desalting of phosphopeptides is typically performed using reverse phase C_{18}-based columns. Therefore, to minimize loss of phosphopeptides, it is important to have fewer desalting steps. With these considerations, we sought to develop an alternative chromatography method

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[1] The abbreviations used are: RP-HPLC, reverse phase-based HPLC; HILIC, hydrophilic interaction chromatography; SCX, strong cation exchange; MMS, methyl methanesulfonate; IgG, immunoglobulin G.

[2] C. P. Albuquerque, M. B. Smolka, S. H. Payne, V. Bafna, J. Eng, and H. Zhou, unpublished observation.

for phosphopeptide fractionation. Ideally such chromatography does not require the use of salt-containing buffers and provides an orthogonal separation compared with RP-HPLC.

Hydrophilic interaction chromatography (HILIC) is a less commonly used method for peptide fractionation despite that it is often used to fractionate small metabolites (14). Interestingly a recent study of the separation of unphosphorylated peptides using SCX, HILIC, and RP-HPLC indicated that a better orthogonal separation could occur between HILIC and RP-HPLC for unphosphorylated peptides (15, 16). The observed orthogonal separation between HILIC and RP-HPLC likely reflects their different mechanisms of separation. Although RP-HPLC depends on the interaction with the hydrophobic amino acid side chains, HILIC depends on the interaction with those hydrophilic and possibly charged amino acid residues via hydrogen bonding and ionic interactions. Moreover because phosphopeptides are generally hydrophilic and charged, one would expect that phosphopeptides should interact more strongly with HILIC than do unphosphorylated peptides. Thus, it should be possible to separate phosphopeptides using HILIC.

Here we investigated the use of HILIC for phosphopeptide separation and found that it provided a largely orthogonal separation of phosphopeptides with RP-HPLC. A multidimensional chromatography technology combining IMAC, HILIC, and RP-HPLC in a sequential order was thus developed for the purification and separation of phosphopeptides. This technology was designed to have minimal manual steps and found to provide an in-depth and sensitive mapping of the phosphoproteome of the yeast *Saccharomyces cerevisiae* after genotoxic stress.

EXPERIMENTAL PROCEDURES

Preparation of IMAC Resin—All commonly used chemicals were obtained from Sigma-Aldrich unless noted otherwise. Resins from three silica-nitrilotriacetic acid spin columns (Qiagen, Valencia, CA) were added to 50 ml of buffer containing 5 mM EDTA (pH 8.0) and 1 M NaCl and incubated for 1 h at room temperature under rotation. The resins were then spun down and washed sequentially with 50 ml of water and 50 ml of 0.6% acetic acid and finally incubated with 50 ml of 100 mM $FeCl_3$ in 0.3% acetic acid for 1 h under rotation. The resins were then washed with 50 ml of 0.6% acetic acid; then with 50 ml of a solution containing 25% acetonitrile, 0.1 M NaCl, and 0.1% acetic acid; and then two more times with 50 ml of 0.1% acetic acid. Finally the resins were resuspended in 0.1% acetic acid as a 50% (v/v) slurry and stored at 4 °C (17).

Purification of Phosphopeptides from Whole Cell Protein Extract—Yeast cells (RDK2669: *MAT*α, *ura3–52*, *leu2*Δ*1*, *trp1*Δ*63*, *his3*Δ*200*, *lys2*Δ*Bgl*, *hom3–10*, *ade2*Δ*1*, *ade8*) were grown in 2 liters of YPD medium until an A_{600} of 0.7, then treated with 0.05% methyl methanesulfonate (MMS) for 3 h, and harvested. Protein extract was prepared by grinding 5 g of yeast cell pellet in an ice-cold bead beater in 40 ml of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2% Nonidet P-40, 5 mM EDTA, 5 mM NaF, 10 mM β-glycerophosphate, and 1 mM phenylmethylsulfonyl fluoride. Cell debris were removed by centrifugation at 30,000 $\times$ *g* for 30 min. SDS was added to a final concentration of 1% along with 5 mM DTT for 5 min at 50 °C. After cooling to room temperature, iodoacetamide was added to a final concentration of 30 mM for 40 min. The proteins were precipitated using 3 volumes of 50% (v/v) ethanol/acetone for 30 min at 4 °C. Precipitated proteins were resuspended with a buffer containing 8 M urea and 100 mM Tris-HCl (pH 8.0), and the protein concentration was measured using the Bradford assay. The sample was then diluted using 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl (TBS) so that the final concentration of urea was below 2 M. Six milligrams of proteins were then digested by 100 μg of trypsin at 37 °C overnight. The proteolyzed sample was acidified using TFA to a final concentration of 0.2% and then spun down for 20 min at 4000 $\times$ *g* to remove any insoluble material. The cleared peptide sample was loaded to a 200-mg Sep-Pak C_{18} column (Waters), washed two times with 3 ml of 1% acetic acid, then eluted by 600 μl of 80% acetonitrile and 0.1% acetic acid, and dried. The dried peptides were resuspended in 100 μl of 1% acetic acid and loaded to a gel loading tip column containing 20 μl of IMAC resin. After loading, the IMAC resin was washed twice with 20 μl of wash buffer containing 25% acetonitrile, 100 mM NaCl, and 0.1% acetic acid and then with 20 μl of 1% acetic acid; finally eluted by 100 μl of 1% phosphoric acid; and dried under vacuum.

Purification of Rad9 and Mrc1 Using a Pulldown Approach—A TAF tag containing two copies of protein A, tobacco etch virus cleavage site, six histidines, and three copies of the FLAG peptide was inserted at the C terminus of either the Rad9 or Mrc1 protein using a G418 selection marker and RDK2669 yeast background as described previously (18). Rad9-TAF and Mrc1-TAF cells were grown in 2 liters of YPD medium until an A_{600} of 0.7, then treated with 0.05% MMS for 3 h, and harvested. Protein extract was prepared the same way as above, and 500 mg of proteins were obtained. Protein extract was incubated with 0.1 ml of human immunoglobulin G (IgG)-Sepharose resin (GE Healthcare) for 4 h in the cold room under rotation. The IgG resin was then washed with 10 ml of lysis buffer and then with 5 ml of TBS. The proteins were eluted from the IgG resin using 800 μl of 5% acetic acid and 1 M urea. The eluted proteins were dried under reduced pressure, and then 1 M Tris base was used to neutralize the sample to pH 8. After the sample volume was adjusted to 100 μl, it was reduced by DTT and alkylated by iodoacetamide as described above. The sample was then diluted with TBS to a final concentration of 2 M urea and digested with 2 μg of trypsin at 37 °C overnight. The resulting peptides were desalted using a 50-mg Sep-Pak C_{18} column (Waters) and dried under vacuum. The dried peptides were resuspended in 100 μl of 1% acetic acid and loaded to a tip column containing 10 μl of IMAC resins. After loading, the IMAC resin was washed twice with 10 μl of wash buffer containing 25% acetonitrile, 100 mM NaCl, and 0.1% acetic acid; then washed with 20 μl of water; finally eluted by 100 μl of 3% ammonium hydroxide; and dried under vacuum.

The Use of HILIC for Phosphopeptide Separation—A TSK gel Amide-80 column (2 mm $\times$ 15 cm, 5 μm; Tosoh, Grove City, OH) was used for the HILIC experiment. Three buffers were used for the gradient: buffer A, 90% acetonitrile and 0.005% TFA; buffer B, 0.005% TFA; and buffer C, 0.1% phosphoric acid and 0.005% TFA. Phosphopeptides eluted from IMAC were resuspended in 30 μl of 90% acetonitrile with 0.1% formic acid and then injected into the HILIC Amide-80 column via a 100-μl loop with a flow rate of 150 μl/min. One and a half-minute fractions from the HILIC were collected and dried under reduced pressure. The gradient used is shown in Fig. 1*B* (100% buffer A at time = 0 min, 11% buffer B at 5 min, 29% buffer B at 20 min, 95% buffer C at 45 min, 95% buffer C at 50 min, and finally 100% buffer A at 55 min).

Mass Spectrometry—Experiments were performed using the 1100 QuadPump HPLC system (Agilent, Santa Clara, CA), the Ultimate 3000 autosampler (Dionex, Sunnyvale, CA), and the LTQ tandem mass spectrometer (Thermo Fischer Scientific, San Jose, CA). Each HILIC fraction was transferred to a silanized glass insert (National

Scientific, Rockwood, TN), dried under reduced pressure, and then resuspended in 10 μl of 0.1% TFA. Four microliters were loaded using the autosampler via a 5-μl sample loop directly to an in-house packed 125-μm (inner diameter) $\times$ 20-cm microcapillary RP-HPLC column packed with 3-μm C_{18} resin (Magic beads; Michrom Bioresources, Auburn, CA). For RP-HPLC-MS/MS analysis, Buffer I consisted of 0.1% formic acid and 2% acetonitrile. Buffer II consisted of 0.1% formic acid and 80% acetonitrile. A 120-min gradient from 15 to 35% Buffer II was used. Xcalibur 2.2 software (Thermo Fischer Scientific) was used for the data acquisition, and the mass spectrometer was set to perform one full MS scan followed by six consecutive MS/MS scans according to the ion intensities detected in the full MS scan. The minimal threshold for the dependent scans was set to 6500 counts, and a dynamic exclusion list was used with the following settings: repeat count of 1, repeat duration of 2 s, exclusion list size of 150, exclusion duration of 60 s, and exclusion mass width of 0.2% relative to the reference mass.

Data Analysis Using SEQUEST and InsPecT—To search tandem mass spectra, a composite database was generated using both the yeast protein database (downloaded from *Saccharomyces* Genome Database on January 12, 2007) and its reverse protein database. The use of such decoy database allows an estimate of the false discovery rate (19). Parameters for the search were: parent mass tolerance of 3.0 Da, +80.0-Da variable modification of STY due to phosphorylation, and a maximum of two modifications per peptide. For the use of SEQUEST (version 3.4 beta 2), a Sorcerer system (SageN, San Jose, CA) was used, and a semitryptic restriction was applied to the search. For the use of InsPecT (version July 12, 2007) (20), no tryptic restriction was used in the search. After the search, the raw results of SEQUEST and InsPecT were ranked according to their provided *p* value and filtered to a 1% false discovery rate as measured by hits to the decoy sequence. The identified phosphopeptides were then filtered to remove redundancy due to different charge states, oxidation, repeated identification, or any possible ambiguity in phosphorylation site assignment so that only the number of phosphopeptides with unique amino acid sequences was reported in Fig. 3. This provides a simple, although underestimated, measure of the number of phosphopeptides identified in this study.

Phosphate Localization Score (PLscore)—To measure confidence in the assignment of phosphorylation site localization, we implemented a metric similar to the Ascore introduced by Beausoleil *et al.* (21) with minor changes. Binomial probabilities for observing peaks supporting a specific phosphate site localization over another were calculated similarly to the Ascore except that a single peak density was used as opposed to the multiple iterative peak densities in the Ascore. With this simplification, we computed a peptide score using the default peak density of InsPecT, 12 peaks per 100 *m/z* units. This resulted in slightly lower scores than in Ascore. The algorithm was applied to both InsPecT and SEQUEST results. This script, named PhosphateLocalization.py, is freely available as part of the InsPecT package.

RESULTS

Experimental Strategy and Rationale—The objective was to develop a multidimensional liquid chromatography technology for global phosphorylation analysis. First, RP-HPLC is the standard tool to fractionate peptides immediately prior to their analysis by tandem MS. Any additional chromatography should provide a separation that is orthogonal to RP-HPLC. Second, for a sensitive detection of phosphopeptides, the use of such chromatography should avoid the use of any desalting step that may lead to sample loss. Third, for a rapid phosphoproteome analysis, manual operations should be minimized. Based on these considerations, a technology was developed for the purification, separation, and analysis of phosphopeptides using MS (see Fig. 1*A*).

As shown in Fig. 1*A*, 6 mg of peptides derived from proteolyzed protein extract were applied to IMAC. One percent phosphoric acid was used to elute phosphopeptides. The eluted phosphopeptides were dried under vacuum, then resuspended in a buffer containing 90% acetonitrile and 0.1% formic acid, and loaded to a HILIC column. The phosphopeptides were then eluted from HILIC using an increasing concentration of aqueous buffers. The eluent from HILIC was dried under vacuum, resuspended in 0.1% TFA, and then loaded for RP-HPLC-MS/MS via an autosampler. TFA was found to be important for loading of phosphopeptides for RP-HPLC because it helps the binding of phosphopeptides to the C_{18} column.

As shown in Fig. 1*B*, a dual gradient program was used for the separation of phosphopeptides by HILIC using volatile buffers without any salt. In the first region of separation (Fig. 1*B*, *gray dashed line*), a gradient of increasing concentration of water with a constant concentration of 0.005% TFA was used. In the second region of separation (Fig. 1*B*, *red dashed line*), a gradient of increasing concentration of phosphoric acid and water was introduced. The elution profiles of phosphorylated (IMAC-bound, *dark blue line*) and the unphosphorylated peptides (IMAC flow-through, *light blue line*) were examined. As shown in Fig. 1*B*, unphosphorylated peptides generally eluted earlier than the phosphorylated peptides, indicating that phosphopeptides bind more tightly to the HILIC column than do unphosphorylated peptides. The partial overlap of the phosphorylated and unphosphorylated peptides indicated that HILIC is not sufficient for phosphopeptide purification; instead the use of IMAC or other affinity methods for phosphopeptide purification is essential. After IMAC purification, a low level of unphosphorylated peptides was often found in the earlier HILIC fractions, which were no longer considered for later analysis (see Fig. 1*B*, *asterisks*). This UV profile of the phosphopeptides on the HILIC was reproducible from multiple experiments (results not shown).

The Use of HILIC to Fractionate Phosphopeptides—To see whether HILIC could provide a high resolution separation of phosphopeptides that is orthogonal to RP-HPLC, the phosphopeptides in HILIC fractions 14–16 were examined. As shown in Fig. 2*A*, numerous peptide ions are present throughout the RP-HPLC gradient for these fractions, underscoring the need for fractionation of phosphopeptides purified from proteolyzed cell lysate. Interestingly each HILIC fraction has a different elution profile on RP-HPLC, and peptides are present throughout the RP-HPLC gradient, indicating an orthogonal separation of phosphopeptides between HILIC and RP-HPLC. The phosphopeptides in HILIC fractions 14–16 were then identified using SEQUEST and analyzed for potential overlaps between the fractions. As shown in Fig. 2*B*, there is

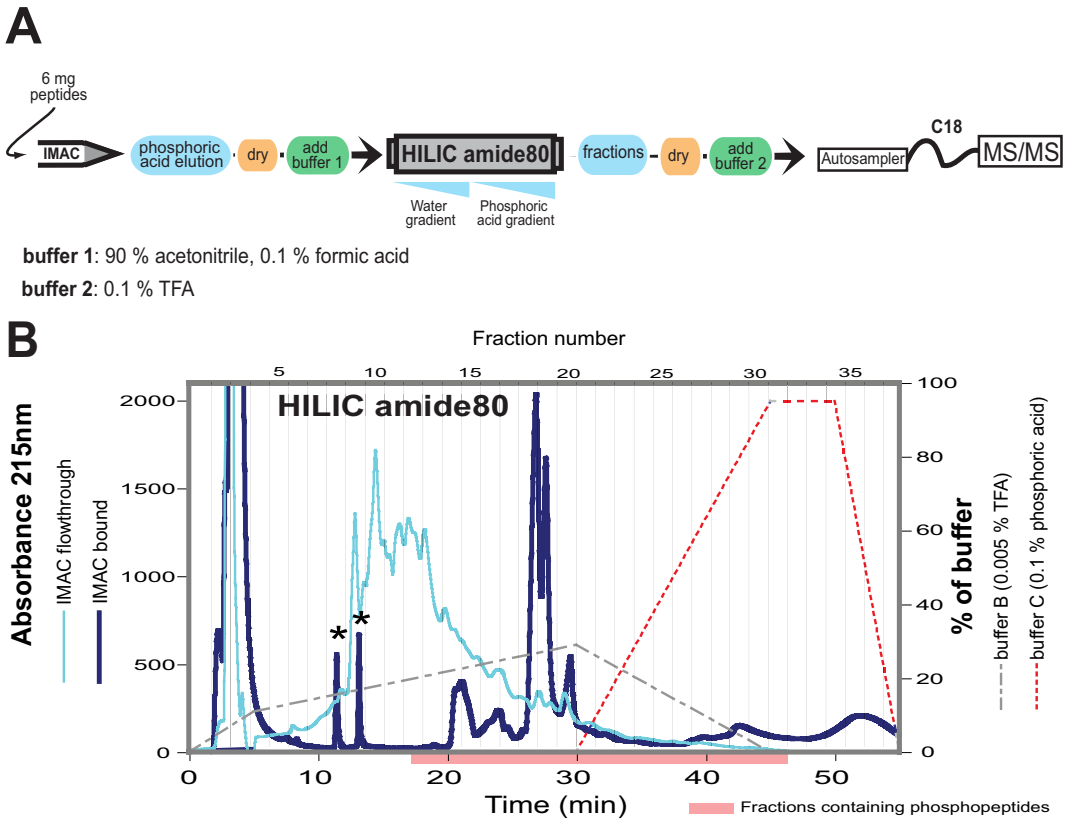

FIG. 1. **The use of HILIC for phosphopeptide separation.** *A*, schematics of a multidimensional chromatography technology used to purify, separate, and analyze the phosphopeptides purified from proteolyzed cell lysate. *B*, the gradients used in HILIC (*dashed lines*) and the UV absorbance of the peptides in the bound fraction (*dark blue line*) and flow-through (*light blue line*) of IMAC separated by the HILIC. There is a partial overlap between the unphosphorylated and phosphorylated peptides. The HILIC fractions from 13–32 containing mostly phosphopeptides, colored in *red*, were analyzed by RP-HPLC-MS/MS. The peak appearing before 10 min is due to injection and contains few peptides. Unphosphorylated peptides were mostly found in the fractions indicated by *asterisks*.

an approximate 20% overlap in the identified phosphopeptides between two adjacent HILIC fractions. Interestingly a small overlap (~5%) was found between the non-neighboring HILIC fractions 14 and 16. Similar results were also observed for other HILIC fractions (not shown). These observations showed that HILIC provides a high resolution separation of phosphopeptides that is largely orthogonal to RP-HPLC.

An In-depth Mapping of the Phosphoproteome of the Budding Yeast after DNA Damage—Next SEQUEST and InsPecT were used to identify phosphopeptides present in 20 HILIC fractions (see Fig. 3*A*) (20, 22). A decoy protein database of the budding yeast *S. cerevisiae* was included in the database search to evaluate the false discovery rate (19). With a false discovery rate of 1%, a total of 8764 unique phosphopeptides (irrespective of their phosphorylation site assignment) from 2278 proteins were identified using both SEQUEST and InsPecT (see Fig. 3*A*). Although SEQUEST was able to identify 6419 unique phosphopeptides, InsPecT identified 7681 phosphopeptides from the same data set. Furthermore 5336 unique phosphopeptides were found by both InsPecT and SEQUEST (see Fig. 3*B*). Thus, SEQUEST and InsPecT are partially complementary for phosphopeptide identification. This is likely because the scoring mechanisms for SEQUEST and InsPecT are quite different (20).

Of all the top matched phosphorylation sites found, serine phosphorylation was most common, and it constituted ~83% of all phosphorylation found, whereas threonine phosphorylation accounted for the rest. Furthermore the number of doubly phosphorylated peptides was much fewer than singly phosphorylated peptides (756 *versus* 8008). This is likely because multiply phosphorylated peptides are harder to identify with high confidence using LTQ-MS because of its low mass resolution and the use of collision-induced dissociation. As commonly observed, tandem mass spectra of phosphopeptides obtained via collision-induced dissociation are often of lower quality due to the neutral loss of phosphoric acid, which compromises the assignment of the precise phosphorylation site within a peptide. Although the top matched phosphopeptides are reported in supplemental Tables 1 and 2, only the number of unique phosphopeptides regardless of the phosphorylation site assignment is summarized here (see Fig. 3, *A* and *B*). Despite being an underestimation, the large number of unique phosphopeptides identified here showed the poten-

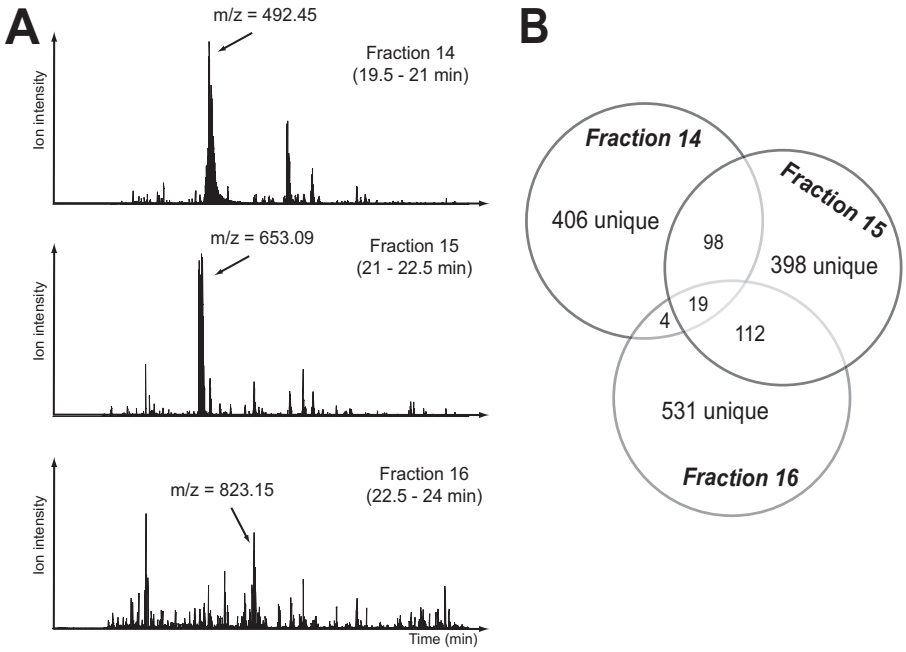

FIG. 2. **Comparison of the RP-HPLC profile and phosphopeptide identification from three adjacent HILIC fractions.** *A*, ion abundances of the peptides detected by RP-HPLC-MS for HILIC fractions 14–16. Numerous peptide ions appear throughout RP-HPLC in each fraction that are different from one another. *B*, Venn diagram indicates the overlaps of the identified phosphopeptides in HILIC fractions 14–16. Although there is an ~20% overlap between adjacent fractions, few overlaps (~5%) were found between fractions 14 and 16.

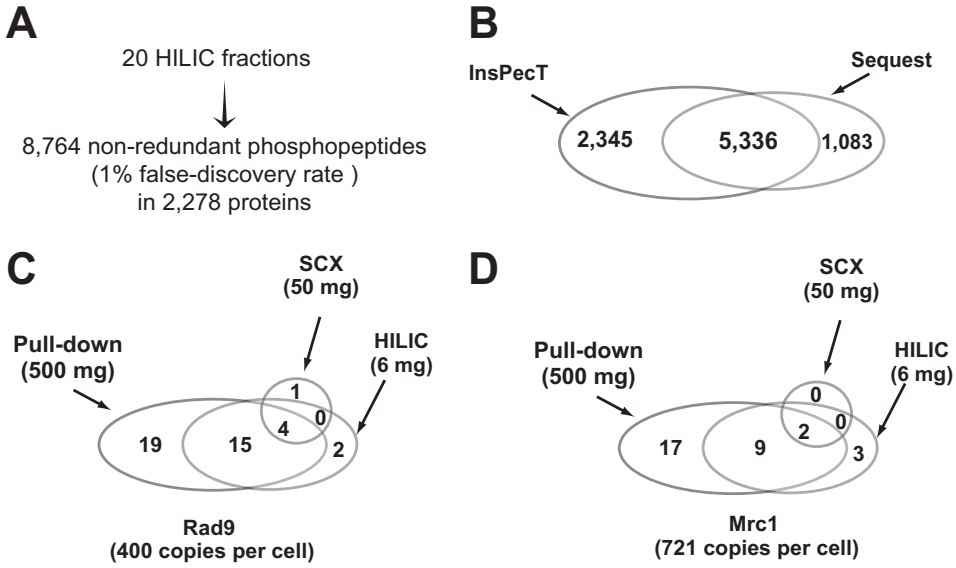

FIG. 3. **Summary of the phosphopeptides and phosphoproteins identified from yeast.** *A*, phosphopeptides were purified using 6 mg of total proteins as a starting material. After separation using HILIC, 20 fractions were analyzed by RP-HPLC-MS/MS and searched using SEQUEST and InsPecT. This led to the identification of 8764 unique phosphopeptides from 2278 proteins. Possible redundancy due to charge state, oxidative state, or even possible ambiguity on phosphorylation site assignment was removed to calculate the number of unique phosphopeptides reported here. The false discovery rate is less than 1% as judged by the target-decoy strategy. *B*, Venn diagram of the phosphopeptides identified using InsPecT and SEQUEST, indicating that these two search tools are partially complementary to each other. *C*, Venn diagram of the number of phosphopeptides of Rad9 identified using the HILIC, SCX, and pulldown approaches. *D*, Venn diagram of the number of phosphopeptides of Mrc1 identified using the HILIC, SCX, and pulldown approaches. The amount of starting material used in each case is indicated. In this case, manual examination of each phosphopeptide of Rad9 and Mrc1 was performed.

tial of this multidimensional chromatography technology for large scale phosphoproteomics studies.

A key question is how well phosphorylation of low abundance proteins was identified using this approach. To address this question, we chose to further examine phosphorylation of Rad9 and Mrc1, two low abundance proteins with estimated 400 and 721 copies per cell, respectively (23). Rad9 and Mrc1 are two key adaptor proteins in the yeast DNA damage and replication checkpoints, and they are known to be phosphorylated in response to MMS treatment (24, 25). However, their phosphorylation sites have not been mapped previously. To identify the most phosphopeptides of Rad9 and Mrc1 using

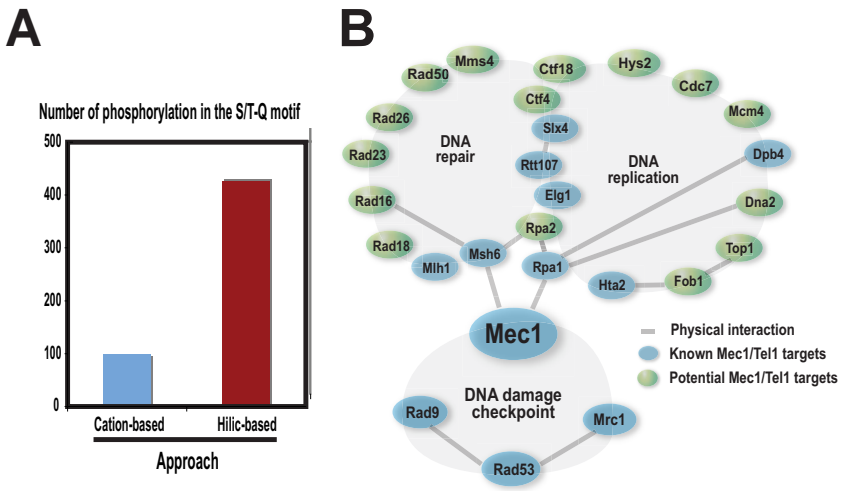

FIG. 4. **Analysis of the SQ/TQ phosphorylation in the budding yeast.** *A*, comparison of the number of SQ/TQ phosphorylated peptides using either the SCX-based or the HILIC-based technology. About 4-fold more SQ/TQ phosphorylated peptides were found here. *B*, a summary of the SQ/TQ phosphorylated proteins with known roles in DNA replication, repair, and DNA damage checkpoint. Those known Mec1/Tel1 targets are colored in *blue*, whereas the newly identified potential targets are colored in *green*.

MS, we purified each protein from 500 mg of protein extract derived from MMS-treated Rad9-TAF and Mrc1-TAF cells using a pulldown approach (17). Phosphopeptides of purified Rad9 and Mrc1 were further purified using IMAC and identified (see supplemental Tables 3 and 4). After database search using SEQUEST and InsPecT as described under "Data Analysis Using SEQUEST and InsPecT," the identified phosphopeptides of Rad9 and Mrc1 were subjected to manual inspection. To this end, all the identified phosphopeptides of Rad9 and Mrc1 were required to be doubly tryptic, and all significant fragment ions in their MS/MS spectra should be correctly assigned. The results are summarized in Fig. 3*C* and supplemental Tables 3 and 4. Although the pulldown approach identified 38 phosphopeptides from Rad9, 19 of them were also identified by the HILIC method. This corresponds to ~50% coverage of the phosphorylation of Rad9. Next the phosphopeptides of Rad9 identified here were compared with those identified using the SCX-based method (8). Despite that almost 8-fold more starting material (50 mg) was used previously (8), more phosphopeptides of Rad9 were found using the HILIC-based method. Similar results were obtained with Mrc1 (see Fig. 3*D*). Thus, this IMAC-HILIC-RP-HPLC-based technology is more sensitive, requires fewer manual efforts, and allows a higher coverage of the phosphorylation of low abundance Rad9 and Mrc1 in cells compared with our previous study (8).

Phosphorylation of SQ/TQ Sites of the Budding Yeast after DNA Damage—As shown previously, phosphorylation of SQ/TQ sites is a relatively rare event in cells (8). Mec1 and Tel1 are responsible for about 26% of the observed SQ/TQ phosphorylation in cells treated with MMS. In the present study, phosphorylated SQ/TQ sites were found in 355 proteins, which is ~4 times the number of proteins identified by the SCX method (8) (see Fig. 4*A* and supplemental Table 4). Fig. 4*B* summarizes the known and potential targets of Mec1/Tel1 that are involved in the DNA damage checkpoint, DNA repair, and DNA replication. Several proteins that were known to undergo DNA damage-induced phosphorylation, including Slx4 (26), Rtt107 (27), Mrc1 (25), H2A (28), and others, were identified here (see Fig. 4*B*, colored in *blue*), most of them were not described previously (see Fig. 4*B*, colored in *green*). Further experiments are in progress to see whether they undergo Mec1- and/or Tel1-dependent phosphorylation *in vivo*. It should also be noted that additional targets of Mec1 and Tel1 are likely present in the database of SQ/TQ phosphorylated peptides (see supplemental Table 5).

DISCUSSION

With the enormously complex phosphoproteome unveiled in several recent studies, a key objective of global phosphorylation analysis is to obtain an in-depth mapping of protein phosphorylation in cells. This is needed to identify and characterize many lower abundance and regulatory phosphorylation events in cells. To this end, a suitable multidimensional chromatography technology should be used to separate phosphopeptides into simpler samples for mass spectrometric analysis. Ideally such technology should be simple, automatable, and allow a sensitive detection of phosphopeptides.

In this study, we described the development of a multidimensional chromatography method based on a combination of IMAC, HILIC, and RP-HPLC to purify and fractionate phosphopeptides. Several features of this technology are demonstrated. First, HILIC was found to be largely orthogonal to RP-HPLC for phosphopeptide separation. Little overlap of phosphopeptides was found between non-neighboring HILIC fractions. As a result, a higher coverage of the phosphoproteome was obtained compared with our previous study (8). During the reviewing of this manuscript, a recent report by McNulty and Annan (29) showed that phosphopeptides bind to HILIC more tightly than do unphosphorylated peptides, and HILIC could be used to fractionate phosphopeptides; this is in agreement with our observation (see Fig. 1*B*). Second, a salt-free buffer system was developed for the use of HILIC to separate phosphopeptides. As a result, the only sample-han-

dling step used here is sample drying and resuspension, which should not cause any sample loss. This is likely the main reason why more phosphopeptides were identified with less starting material compared with our previous study (8). Third, because the elution buffer of HILIC contains organic solvent (acetonitrile), an off-line strategy must be used between HILIC and RP-HPLC. Following sample drying and resuspension, the use of an autosampler permits the analysis of many samples continuously without manual intervention. Finally this technology should be compatible with various stable isotope labeling methods for quantitative analysis of protein phosphorylation (17, 30, 31).

Comparison of the phosphopeptides of Rad9 and Mrc1 detected by this multidimensional chromatography technology and the conventional pulldown method showed that ~50% of the phosphorylation of Rad9 and Mrc1 was obtained from ~1% of the starting material used in the multidimensional chromatography technology. Compared with the SCX-based method, this HILIC-based technology appears to be more sensitive and allows a higher coverage of phosphoproteome and importantly greater ease of use. Furthermore this study allowed us to generate a more complete database of the SQ/TQ phosphorylation motifs that is expected to contain the potential substrates of Mec1 and Tel1. We suggest that many of these newly identified proteins, with SQ/TQ phosphorylation and known roles in DNA replication and repair, could be directly phosphorylated by Mec1 and/or Tel1.

Recently it was reported that the use of hybrid instruments, which combine the high resolution of an FT-ICR detector with the high rate of MS/MS acquisition of a linear ion trap, could lead to a 3-fold increase in the number of identified phosphopeptides as compared with the LTQ-MS instrument used here (32). By combining the multidimensional chromatography method described here with high mass resolution MS instruments, we anticipate that an even higher coverage of the phosphoproteome could be achieved.

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