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Global Phosphoproteome of HT-29 Human Colon Adenocarcinoma Cells

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Phosphorylation events in cellular signaling cascades triggered by a variety of cellular stimuli modulate protein function, leading to diverse cellular outcomes including cell division, growth, death, and differentiation. Abnormal regulation of protein phosphorylation due to mutation or overexpression of signaling proteins often results in various disease states. We provide here a list of protein phosphorylation sites identified from HT-29 human colon adenocarcinoma cell line by immobilized metal affinity chromatography (IMAC) combined with liquid chromatography (LC)-tandem mass spectrometry (MS/MS) analysis. In this study, proteins extracted from HT-29 whole cell lysates were digested with trypsin and carboxylate groups on the resulting peptides were converted to methyl esters. Derivatized phosphorylated peptides were enriched using Fe^{3+}-chelated metal affinity resin. Phosphopeptides retained by IMAC were separated by high performance liquid chromatography (HPLC) and analyzed by electrospray ionization-quadrupole-time-of-flight (ESI-Q-TOF) mass spectrometry. We identified 238 phosphorylation sites, 213 of which could be conclusively localized to a single residue, from 116 proteins by searching MS/MS spectra against the human protein database using MASCOT. Peptide identification and phosphorylation site assignment were confirmed by manual inspection of the MS/MS spectra. Many of the phosphorylation sites identified in our results have not been described previously in the scientific literature. We attempted to ascribe functionality to the sites identified in this work by searching for potential kinase motifs with Scansite (http://scansite.mit.edu) and obtaining information on kinase substrate selectivity from Pattern Explorer (http://scansite.mit.edu/pe). The list of protein phosphorylation sites identified in the present experiment provides broad information on phosphorylated proteins under normal (asynchronous) cell culture conditions. Sites identified in this study may be utilized as surrogate bio-markers to assess the activity of selected kinases and signaling pathways from different cell states and exogenous stimuli.

Keywords: phosphoproteome • IMAC • HT-29 • scansite • pattern explorer

Introduction

Phosphorylation is a crucial post-translational modification which regulates the function of proteins involved in signaling pathways. Aberrant regulation of phosphorylation, accordingly, has been associated with diverse disease states.[1,2] For instance, abnormal activity of protein kinases and phosphatases due to their overexpression or mutation has been reported in multiple cancer studies.[2] To develop a better understanding of the mechanisms involved in these various disease states, mapping of the components and regulatory events in signaling pathways, such as kinases, phosphatases, and their substrates, particularly oncogenes or tumor suppressor genes, has been extensively studied.

Traditionally, ^{32}P-based protein blotting and phosphopeptide mapping combined with Edman sequencing have been used to visualize phosphoproteins and identify modification sites, respectively. Although these procedures provide information regarding protein phosphorylation state, they also require intensive labor and radioactive materials. More recently, the task of mapping protein phosphorylation sites from complex biological samples has been greatly facilitated by several methodological improvements aimed at efficient isolation of phosphorylated peptides in order to overcome ionic suppression of phosphorylated peptides in the presence of an abundance of nonphosphorylated peptides. In some of the earlier efforts to enrich for phosphorylated peptides, Oda et al. isolated and identified phosphopeptides by labeling with biotin following β-elimination,[3] while Zhou et al. captured phosphopeptides by covalent bonds to glass beads after adding sulfhydryl to phosphate moieties.[4] These methods enhanced selectivity, but required multistep sample preparation and detected only high-abundance proteins possibly due to sample loss from harsh experimental conditions. To overcome the labile nature of the phosphate group during collision activated dissociation (CAD) while enriching for phosphorylated peptides, Knight et al. introduced chemical transformation of phospho-serine/threo-

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nine into lysine analogues using β-elimination followed by a reaction with aminoethylcysteine, enabling phosphospecific cleavage, unique y_1 ions, and consequently improved MS/MS data interpretation.[5] This technology cannot distinguish phosphorylation from O-glycosylation unless combined with phosphatase or glycosidase pretreatment. Also, this method is still limited to only phospho-serine/threonine residues and has not been applied to biological samples yet. Several groups have also utilized pan-specific anti-phospho antibodies to enrich for selected subclasses of phosphorylated proteins and peptides prior to MS analysis.[6−8] This method has typically been limited to enrichment of proteins and peptides phosphorylated at tyrosine residues, which comprise only 0.05% of all phosphorylated proteins,[9] and therefore does not provide broad information on the entire phosphoproteome. Despite recent improvement, phosphoserine/threonine proteins are still difficult to analyze with this approach due to lack of efficient antibodies.[10] Mapping of tyrosine phosphorylation sites has also been demonstrated with precursor ion scanning (for m/z 216.043, the phosphotyrosine immonium ion) in mass spectrometry analysis, but this method is also not applicable to phosphoserine/threonine residues.[7,11−15] Annan et al. also introduced a multidimensional electrospray mass spectrometry approach to map phosphopeptides based on the production of phosphopeptide-specific marker ions, m/z 63 and/or 79 in the negative mode.[16] This method is applicable to serine, threonine, or tyrosine phosphorylation, but requires switching to positive ion mode for peptide sequencing from MS/MS fragmentation.

Several chromatographic approaches to phosphopeptide enrichment have also been demonstrated. Ficarro et al. combined nano liquid chromatography (LC)/mass spectrometry (MS) analysis and immobilized metal affinity chromatography (IMAC), which was developed and applied by various groups.[17−24] to characterize phosphopeptides from yeast.[25] In this work, conversion of carboxylate groups into methyl esters reduced nonspecific binding to IMAC, thereby yielding a higher coverage of the phosphoproteome including low-abundance phosphoproteins from *S. cerevisiae*. Notably, this IMAC−LC−MS/MS method involved only a single step and detected all three phosphorylation forms. Nuhse et al. utilized strong anion exchange (SAX) to fractionate and enrich for phosphorylated peptides prior to a second enrichment step by IMAC,[19] while Beausoleil et al. utilized strong cation exchange chromatography (SCX) at pH 2.7 to enrich for phosphorylated peptides based on a decrease in retention time due to the negatively charged phosphate at this pH.[26] Most recently, Gruhler et al. combined metabolic stable isotope labeling, enrichment and fractionation by SCX, IMAC, and LC−MS/MS to analyze and quantify yeast protein phosphorylation sites following pheromone stimulation.[27]

HT-29, a human colon adenocarcinoma cell line, has been used as a model system to predict cell decision processes based on regulation of kinase activity in response to diverse cellular stimuli.[28,29] Although selected kinase activity profiles have proven to be quite effective in predicting response to future stimuli, monitoring of additional signaling events will extend our knowledge of the mechanisms underlying cell decision processes. To generate information regarding the phosphorylation state of proteins implicated in signal transduction cascades within this cell line, we utilized Fe (III)-charged IMAC to enrich for phosphorylated peptides from HT-29 whole cell lysate and analyzed these peptides by nano-LC−MS/MS. Here, we provide a profile of phosphoproteins and their modification sites from asynchronous HT-29 cells under normal cell culture conditions. Many of the phosphorylation sites identified in this study may be used in future work as bio-markers of kinase and signaling pathway activation state.

Experimental Section

1. Cell Culture. Human colon epithelial adenocarcinoma cell line, HT-29 (provided by the Sorger laboratory in the MIT Biology Department), was maintained in McCoy's 5A medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and L-glutamine (Life Technologies) at 37 °C under 5% CO_2. Cells were seeded onto culture plates at a density of $5 \times 10^4/cm^2$ and grown for 24 h.

2. Protein Extraction, Digestion, and Peptide Chemical Modification. Proteins were extracted from 5×10^6 HT-29 cells using Trizol (Life Technologies) according to the manufacturer's instruction. Following extraction, the protein pellet was resolubilized in 1% of SDS and diluted to 0.2% of SDS with 100 mM ammonium acetate (pH. 8.9). A small aliquot was extracted for protein assay and the remaining proteins were digested with 20 μg of trypsin (Promega, Madison, WI) at 37 °C overnight. Digested peptides were dried in a vacuum centrifuge and carboxylate groups were converted to methyl esters with methanolic HCl (40 μL of thionyl chloride added dropwise to 1 mL of anhydrous methanol) for 2 h followed by drying in a vacuum centrifuge overnight.

3. Enrichment of Phosphopeptides by IMAC. IMAC columns were prepared as described.[25] Briefly, a 15 cm long (750 μm o.d. × 530 μm i.d.) microcapillary fused-silica column (Polymicro Technologies, Phoenix, AZ) was packed with POROS 20 MC in water (Applied Biosystems, Framingham, MA) and washed with 100 mM of [ethylenedinitrilo]-tetraacetic acid (EDTA) (10 min at 10 μL/min). After rinsing with water to remove EDTA, the column was charged with 100 mM $FeCl_3$ for 10 min at 10 μL/min. Excess $FeCl_3$ was removed by rinsing with 0.1 M acetic acid and 20% of the derivatized peptides (corresponding to 1×10^6 cell or ~200μg of total protein equivalents) were loaded into the IMAC column at 1−2 μL/min. Nonphosphorylated peptides were removed by washing with organic buffer comprised of 25% acetonitrile, 100 mM NaCl, and 1% acetic acid. The column was equilibrated with 0.1% acetic acid (10 min at 10 μL/min) and phosphopeptides were eluted with 250 mM of NaH_2PO_4 (pH 8.0) into an 8 cm long (360 μm o.d. × 100 μm i.d.) microcapillary fused silica precolumn packed with 10 μm C18 (YMC, Wilmington, NC).

4. Mass Spectrometry Analysis. After rinsing to remove excess phosphate buffer, the precolumn was connected to a 10 cm long (360 μm o.d. × 50 μm i.d.) microcapillary fused silica analytical column (packed with 5 μm C18 (YMC ODS-AQ, Waters, MA)) with an integrated electrospray ionization tip (~1 μm). Peptides were eluted with a gradient of 0−60% B in 200 min and 60−100% B in 10 min (B = 70% acetonitrile, 0.2 M acetic acid) and electrosprayed directly into a quadruple-time-of-flight mass spectrometer (QSTAR XL, Applied Biosystems, Framingham, MA) with a flow rate less than 50 nL/min. The instrument was run in positive ion mode and cycled through acquisition of a full-scan mass spectrum (m/z 400−1500) (1 s) followed by 3 MS/MS scans (2 s each) sequentially on the three most abundant ions present in the full scan mass spectrum.

5. Database Analysis. All MS/MS spectra were searched against a human protein database with the MASCOT database searching algorithm. Search parameters contained a variable

modification of +80 Da on serine, threonine, and tyrosine; variable neutral loss from phosphorylated serine and threonine amino acids, and a fixed modification of +14 Da on the C-terminus of peptides and the side chains of aspartic acid and glutamic acid. Phosphorylation sites and peptide sequence assignments contained in MASCOT search results were validated by manual confirmation from raw MS/MS data. Briefly, phosphorylated peptides possessing a MASCOT peptide score ≥20 in which the majority of the fragment ions were automatically assigned to y- or b-type fragment ions were printed out. Manual confirmation was based on fragment ion assignment, loss of phosphorylation from y- and b-type ions containing a phosphorylated residue, relative fragment ion intensity N-terminal to proline and glycine, and internal fragment ion assignment where appropriate. Each confirmed phosphoprotein was searched with Scansite (http://scansite.mit.edu) for potential kinase motifs with high, medium, and low stringency and the data set was processed by Pattern Explorer (http://scansite.mit.edu/pe) with high stringency.

Results and Discussion

In this study, we have identified a total of 238 phosphorylation sites from two analyses of HT-29 human colon adenocarcinoma cells. Experimentally, the methodology used to generate these phosphorylation sites includes slight but significant modifications to the methodology utilized by Ficarro et al. to investigate the *S. cerevisiae* phosphoproteome.[25] For instance, following isolation of proteins with Trizol and tryptic digestion to peptides, carboxylate groups were modified to methyl esters using methanolic HCl composed of thionyl chloride and methanol instead of acetyl chloride and methanol. The thionyl chloride-based reaction is more vigorous as compared to the acetyl chloride-based reaction, typically proceeds to completion in a single reaction step, and is more resistant to minute levels of water contamination. Another significant change was to increase the inner diameter of the IMAC column from 100 μM to 530 μM, corresponding to a 28-fold increase in bed volume for the same bed length, and resulting in decreased competition for binding, especially relevant for the increased levels of protein phosphorylation found in mammalian cell lines as compared to *S. cerevisiae*. Overall, as demonstrated in Figure 1, the method is fairly straightforward, requiring only one chemical modification step, no dialysis (SDS is not retained by the IMAC column, so does not affect downstream LC−MS/MS analysis), a single phosphopeptide purification step, and a 3-hour LC−MS/MS analysis. Due to this simple (no fractionation at the protein or peptide level) sample preparation, the cataloging of protein phosphorylation sites is not as extensive as those recently reported by other groups using additional preparation steps.[26,27] However, the lack of experimental complexity and potential for higher throughput in this work should facilitate subsequent interrogation of protein phosphorylation states following different exogenous stimuli. In our hands, the IMAC−LC−MS/MS method is relatively reproducible; approximately 60% of the phosphorylated peptides from a given analysis will be identified in a replicate analysis (data not shown). Failure to identify the same peptides in subsequent analyses is most likely due to operating the mass spectrometer in information-dependent acquisition mode. In this mode, the mass spectrometer automatically selects the most abundant ions in each full scan mass spectrum for MS/MS fragmentation. The same lower abundance phosphorylated peptides may not be selected for MS/MS fragmentation in each analysis, leading to variation in the peptides (and phosphorylation sites) identified in any pair of replicate analyses.

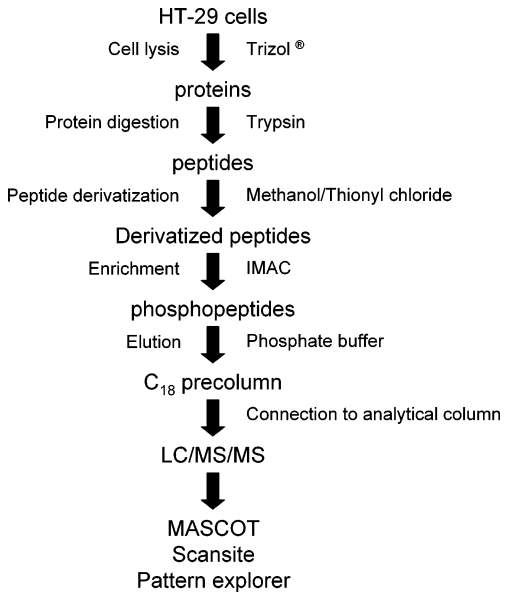

Figure 1. Flowchart of IMAC−LC−MS/MS experiments. Whole HT-29 cell protein extracts prepared with Trizol were digested with trypsin followed by the conversion of carboxylate groups to methyl esters. Derivatized phosphopeptides were then enriched using immobilized metal affinity chromatography (IMAC) and eluted into a reverse-phase (C18) precolumn. Phosphopeptides were, in turn, separated by high performance liquid chromatography (HPLC) and analyzed by electrospray ionization-quadrupole-time-of-flight (ESI-Q-TOF) mass spectrometry. Raw MS/MS data were processed for database search with MASCOT algorithm followed by manual confirmation of phosphorylation site and peptide sequence assignment. Identified peptides and phosphorylation sites were loaded into Scansite to identify potential kinase motifs and Pattern Explorer to identify kinase substrate selectivity.

The entire dataset of protein phosphorylation sites identified in this work are listed in Supplementary Table 1 as Supporting Information. For each of the phosphorylated peptides identified in this work, a MASCOT database search peptide score of at least 20 was obtained; peptide sequence and localization of phosphorylation site were manually confirmed. In addition to the confirmed list of phosphopeptides, we could not conclusively assign some phosphosites or sequences due to insufficient MS/MS data although they showed neutral loss of phosphate group and partial fragmentation information. To give an estimate of the false positive rate from the MASCOT search results, 11.5% of the peptide 'hits' were summarily rejected due to large mass errors (>0.4 Da), while 36% of the peptides with score >20 were rejected due to inconclusive MS/MS spectra.

As expected in an investigation of the global phosphoproteome, many of the phosphopeptides characterized in this study are from high-abundance nuclear and cytosolic proteins involved in structural maintenance or basic cellular biochemistry such as transcription, mRNA processing, and translation (see supplementary Table 1). As detailed in Table 1, phosphorylation sites on proteins known to be involved in signaling pathways were also contained in the data set, including p53, CDC 2 isoform 1, MAPK 14 (p38), Protein kinase C (D2 type), AMP-activated protein kinase α 1 catalytic subunit, AP2 as-

Table 1. Selected Phosphopeptides List from HT-29 Cells[a]

protein	peptides	phosphosites	potential kinases (scansite and Expasy)
apoptosis inhibitor 5 1 (gi|5729730; Q9Y4J7)	ASEDTTSGpSPPKK	S464	(s) casein kinase 1/Cdc2 kinase/ Cdk 5 kinase SEDTTSSPPKK
AP2 associated kinase 1 (gi|29570780)	VGSLpTPPS*SPKTQR	T620	
catenin (cadherinassociated protein), delta 1 (gi|10835010; O60716)	GSLApSLDpSLR	S349, S352	
cell division cycle 2 protein isoform 1 (gi|4502709; P06493)	IGEGpTYGVVYKGR IGEGpTpYGVVYKGR	T14 T14, Y15	Inactivates the enzyme before mitosis Inactivates the enzyme before mitosis Y15: a key role in the radiation-induced G(2) delay (s) Lck kinase IGEGTYGVVYKGR
epidermal growth factor receptor pathway substrate 8-like protein 2 (gi|21264616; Q99K30)	GGDpSPEAK	S217	
KH domain containing, RNA binding, signal transduction associated 1 (gi|5730027; Q07666)	SGpSMDPSGAHPSVR	S20	(s) Akt SGRSGSMDPSGAH
G protein-coupled receptor kinase-interactor 1 (gi| 41393573; Q9Y2 × 7)	HGSGADS*DpYENTQSGDPLLGLEGKR	Y598	(s/l) casein kinase 2 HGSGADSDYENTQS
hepatoma-derived growth factor (gi|4758516; P51858)	RAGDLLEDpSPKRPK	S165	(s/m) Cdc2 kinase AGDLLEDSPKRPK
IAP-associated factor VIAF1 (gi|51492730; Q9H2J4)	RDpSDpSEGD	S271, S273	
mitogen-activated protein kinase 14 isoform 1 (gi|4503069; Q16539)	HTDDEMpTGpYVATR	T179, Y181	
MLL septin-like fusion (gi|5729933; Q9Y5W4)	HVDSLSQRpSPK	S67	
nuclear ubiquitous casein and cyclin dependent kinase substrate (gi|12232387; Q9H1E3)	SGKNpSQEDpSEDSEDKDVK SGKNSQEDpSEDpSEDKDVK	S50, S54 S54, S58	(s) DNA PK SGKNSQEDSEDS (s) Casein kinase 2 GKNSQEDSEDSEDKD
PDGFA associated protein 1 (gi|7657441; Q13442)	KSLDpSDEpSEDEEDDYQQKR	S60, S63	(s) casein kinase 2 KSLDSDESEDEEDDY
protein kinase, AMP-activated, α 1 catalytic subunit (gi| 40254831; Q13131)	SGpSVSNYR	S487	
protein kinase C, D2 type (gi|19923468; Q9BZL6)	RLpSpSTSLASGHSVR	S197, S198	(s) Akt RLSSTSLASG
SH3-domain kinase binding protein 1 (gi|13994242; Q96B97)	ANpSPSLFGTEGKPK	S587	(s/l) calmodulin dependent kinase 2 ANSPSLFGTE
stathmin 1 (gi|5031851; Q96CE4)	ASGQAFELILpSPR	S25	(s/m) Cdk 5 kinase/Cdc 2 kinase QAFELILSPRSKESV
TBC1 domain family, member 4 (gi|7662198; O60343)	GRLGpSVDSFER	S588	
tumor protein p53 (gi|8400738; P04637)	HKKLMFKTEGPDpSD	S392	Casein kinase II; binding to 5.8 rRNA

[a] (s) scansite search with high stringency, (s/m) scansite search with medium stringency, (s/l) scansite search with low stringency. Protein name (Ncbi; Swiss-Prot-trEMBL). *means phosphorylation site without conclusive evidence from manual confirmation.

sociated kinase-1, δ catenin, apoptosis inhibitors, EGFR pathway substrate 8, and PDGFA associated protein-1. As detailed below, detection and identification of phosphorylation sites on these proteins can be directly related to the activation state of various kinases and signaling pathways in the cell.

Cell division cycle 2 protein (CDC2;Cdk 1, p34), a nuclear kinase, plays a key role in cell cycle, mediating transition into mitosis via modulation of phosphorylation sites.[30,31] For instance, phosphorylation of Tyr-15 is absent during the G1 phase, but has been detected during DNA synthesis and reaches maximal level during the G2 phase. At the G2/M boundary, CDC2 becomes dephosphorylated at both Thr-14 and Tyr-15, resulting in entry into mitosis.[31−37] Phosphorylation at another site in the protein, Thr-161, occurs at entry into mitosis and further affects the activation state of this protein.[38,39] In this analysis, we identified peptides with phosphorylation at both Thr-14 and Tyr-15 or Thr-14 alone from HT-29 cells under normal (asynchronous) conditions. In addition to cell cycle associated phosphorylation of these sites, it is possible that the level of phosphorylation could have been elevated due to overexpression of other upstream signaling effectors. For instance, elevation of phosphorylation at Tyr-15 was observed in ErbB2-overexpressing breast primary tumors and cancer cells.[40] An MS/MS spectrum of the doubly phosphorylated peptide from CDC2 is shown in Figure 2.

p53, a tumor suppressor, shows diverse cellular functions such as growth arrest, apoptosis, and cell senescence via its function as a transcription factor. p53 contains multiple phosphorylation sites whose regulation either activates or inactivates the protein. In this work we were able to identify

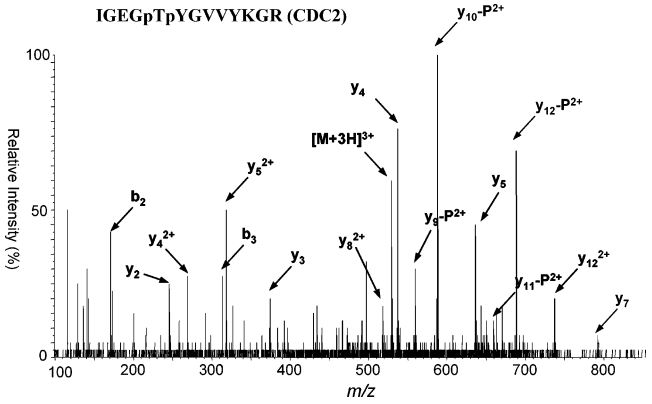

Figure 2. MS/MS spectrum of doubly phosphorylated peptide from cell division cycle 2 protein. MS/MS spectrum resulting from isolation and fragmentation of the triply charged precursor ion of the doubly phosphorylated peptide IGEGpTpYGVVYKGR from CDC2. Note the high sequence coverage generated by singly- and doubly charged y-type fragment ions which enable confident localization of phosphorylation sites within the peptide sequence.

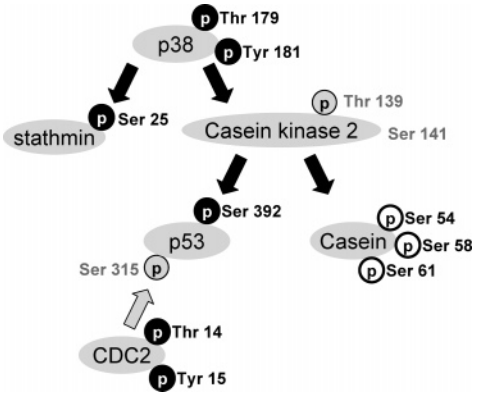

Figure 3. Examples of kinases and substrates in the p38 pathway characterized in our data set. Schematic representation of a portion of the p38 MAP kinase pathway. Several phosphorylation sites on these proteins are known in the literature and confirmed in our data (black circle), or observed in our data without definite evidence to localize phosphorylation sites (gray circles), or unidentified in our data (no circles). We were also able to identify several phosphorylation sites which have not been described in the literature (white circles).

phosphorylation at Ser-392. Interaction of the carboxyl terminus with the middle part of p53 has been reported to prevent p53 from DNA binding.[41] Phosphorylation at Ser-392, a carboxyl terminus residue, results in site-specific DNA binding due to neutralization of the inhibitory basic domain, thereby increasing the formation of functional tetramer and consequent transcription activation.[42−48] Ser-392 phosphorylation might also regulate carboxyl terminus interaction with TBP or p300 by binding within overlapping but different regions from those involved in transactivation, thereby affecting the repression function of p53.[45,49] In response to different cellular signals, complex regulation at a single phosphorylation site most likely requires co-regulatory molecules to generate the different outcomes which have been reported. Phosphorylation of Ser-392 has been ascribed to casein kinase 2 in response to UV radiation, but not to γ-irradiation or a DNA damaging agent, etoposide.[50−55] Compared to normal human cells, human tumor derived cells with mutated p53 showed increased level of phosphorylation at Ser-392,[56] which might be consistent with our characterization of the phosphorylation site in p53 from our system, human colon adenocarcinoma cell line, HT-29, which contains an R to H mutation at position 273 in p53.[57] Additionally, CDC2 was reported to phosphorylate p53 on Ser 315.[58] This site was listed in the MASCOT database search results (contained within the tryptic peptide 306 RALPNNTSpSPQPK 319 from p53) with peptide score 24, but there was not enough fragmentation evidence to conclusively confirm the identity of this peptide and phosphorylation site.

In addition to p53, phosphorylation sites on several other apoptosis associated proteins were also identified in this analysis. Viral inhibitor of apoptosis protein-associated factor-1 (VIAF-1) modulates the activation of caspases during apoptosis.[59] VIAF-1 regulates caspase activity positively, but does not function as an antagonist of inhibitor of apoptosis protein (IAP).[59] Neither its phosphorylation as a regulatory mechanism nor phosphorylation site has been reported. We characterized a phosphopeptide from the C-terminus of VIAF-1 (232 RDpSDpSEGD 239). Considering its co-regulatory function during apoptosis, modulation of phosphorylation at the identified sites could modify the function of VIAF-1. We also characterized a phosphopeptide of another apoptosis-related protein, apoptosis inhibitor-5 (456 ASEDTTSGpSPPKK 468). KH domain containing, RNA binding, signal transduction associated 1, also named Sam68, was reported to be associated with p21 ras and phosphorylated on tyrosine by Src-1 kinase.[60−63] This protein is involved in multiple cellular functions, including transcription, splicing, translation, signal transduction, and cell cycle progression. Overexpression of this protein results in cell cycle arrest and apoptosis.[64] Clearly, identification of another phosphorylation site (Ser-20 in our data set, Thr-59 has been previously characterized) on this protein may help to understand regulatory events affecting the diverse functionality of this protein.

Mitogen-activated protein kinase 14 isoform 1 (also known as p38 MAP kinase) is activated by environmental stress, proinflammatory cytokines, and lipopolysaccharide (LPS). Dual phosphorylation on Thr-179 and Tyr-181 within the conserved Thr-Gly-Tyr site activates p38 MAP kinase,[65] which subsequently phosphorylates downstream kinases and transcription factors.[66,67] In our analysis of HT-29 cells, we characterized both Thr-179 and Tyr-181 phosphorylation sites on p38 MAP kinase as well as phosphorylation of several proteins downstream of this activated kinase. For instance, activation of p38 results in activation of casein kinase 2 and subsequent phosphorylation of p53 at Ser-392 (as mentioned above).[68,69] Although casein kinase 2 appeared in the MASCOT search results, conclusive evidence for peptide identification and phosphorylation site assignment was lacking, so we did not include it in our final list. We identified phosphorylation of stathmin Ser-25, a residue phosphorylated by p38δ in a previous report.[70] Stathmin is a known substrate of p38 MAP kinase and a cytoplasmic protein involved in microtubule dynamics. These phosphorylation events and kinase-substrate relationships (as previously described in the literature) are summarized in Figure 3.

AMP-activated protein kinase (AMPK), a metabolic stress-sensing protein kinase, is responsible for cellular energy status by regulating fatty acid and cholesterol synthesis and glucose homeostasis via protein phosphorylation, thereby switching off biosynthetic pathways when cellular ATP levels are depleted and 5′-AMP rises in response to fuel limitation and/or hypoxia.[71−74] Phosphorylation on Thr-172 in α subunit by AMPKK has been known to activate AMPK.[71,75] We character-

A

-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7
S (14.7)	K (17.2)	R (25.0)	R (18.1)	R (30.2)	S (14.7)	D (13.8)		P (36.2)	S (13.8)	S (16.4)	E (12.1)	S (18.1)	S (17.2)	S (14.7)
R (12.9)	R (13.8)	K (12.1)	K (12.1)	S (14.7)	pS (11.2)	P (10.3)		D (12.1)	E (12.9)	E (10.8)		D (12.1)	G (12.1)	T (10.3)
K (12.1)	S (13.8)				E (11.2)				pS (11.2)					K (10.3)
G (11.2)					R (10.3)				D (10.3)					

B

Motif Group Name	Number of Sites (% of Sites with the Current Pattern)
Phosphoserine/threonine binding group	0 (0.0%)
Tyrosine kinase group	0 (0.0%)
Src homology 2 group	0 (0.0%)
Src homology 3 group	0 (0.0%)
Basophilic serine/threonine kinase group	15 (12.9%)
DNA damage kinase group	1 (0.9%)
Acidophilic serine/threonine kinase group	8 (6.9%)
Proline-dependent serine/threonine kinase group	6 (5.2%)
Kinase binding site group	1 (0.9%)
PDZ domain binding group	0 (0.0%)
Phosphotyrosine binding group	0 (0.0%)
Lipid binding group	0 (0.0%)
User-defined input motif	0 (0.0%)
Sites without hits	86 (74.1%)

Figure 4. Application of Pattern Explorer and Scansite to HT-29 phosphoproteome data set. A. Amino acid abundance percentage information from HT-29 phosphoproteome data set. Data set was processed with high stringency (0.2%) according to instructions in Pattern Explorer and a summary table of abundant (more than 10% in each position) amino acids adjacent to phosphorylation sites (0 position) was prepared. Numbers in parentheses represent abundance percentage of each amino acid. Predominance of proline in position +1 and basic residues in positions −3, −4, −5 may be due to increased activity of proline-directed and basophilic kinases in these cancer-derived cells under standard cell culture conditions. B. Scansite motif group summary view from Pattern Explorer. Phosphorylation sites in the data set were grouped according to kinase motifs. Although most (74%) of the sites do not match to one of the 26 kinase motifs in Scansite, the remaining sites match predominantly to the basophilic kinase group.

ized an unknown phosphorylation site, Ser-487, in the C-terminus involved in complex formation with β and γ subunits.[76]

Several other proteins involved in cellular signaling cascades were identified in this study. For instance, protein kinase D2, or protein kinase C, type D2, was reported to autophosphorylate Ser-876 with stimulation of phorbol esters.[77] Also, Bcr/Abl induced tyrosine phosphorylation in protein kinase D2, resulting in NF-κB activation.[78] We identified two unknown phosphorylation sites on protein kinase D2, S197, and S198, from HT-29 cells. Although the function of these two sites has not been determined, their identification in this study may facilitate future functional analysis. Kinases responsible for the regulation of adaptor protein-2 in adaptor protein complexes (APs), involved in clathrin trafficking and endocytosis, were suggested[79] and later adaptor-associated kinase 1 (AAK), a member of the Prk/Ark family of ser/thr kinases, was copurified with AP2.[80] We identified a phosphorylation site of AAK at T620. G protein-coupled receptor kinase-interactor 1 (GIT1), an ADP ribosylation factor GTPase-activating protein, is involved in epidermal growth factor, angiotensin, and thrombin signaling and cell migration.[81−84] Thrombin was reported to induce tyrosine phosphorylation in GIT1 by using anti-phosphotyrosine antibody.[82] We identified phosphorylation at Tyr-598 in this protein. Also in our list is a phosphorylation site (729 KLPpSTTL 735) in ribosomal protein S6 kinase α 1. This site appears to be due to autophosphorylation by the N-terminal kinase domain; the effect of this site on protein function has not been well-characterized.[85]

We have also processed all of these sites through Pattern Explorer,[86] a new interface to the Scansite motif comparison algorithm (available at http://scansite.mit.edu).[87] As demonstrated in Figure 4, Pattern Explorer is able to process large data sets of protein phosphorylation sites and present information at a variety of levels. At the dataset level, patterns of phosphorylation sites and their surrounding residues can be displayed, facilitating the identification of overriding patterns resulting from either activation of kinases in the sample or potentially from analytical bias in the data set. The table in Figure 4a summarizes the most common amino acids (occurring in a particular position in more than 10% of the phosphorylated peptides) adjacent to phosphorylation sites. From the percentage utilization of each amino acid at each position, it should be possible to draw inferences to the endogenously activated kinases in HT-29. For instance, 36% of phosphorylated peptides in these dataset contain a proline in position +1, potentially reflecting increased activation of proline directed serine/threonine kinases, such as ERK1, CDK5, CDC2, and CDK2, which have demonstrated proline preference at position +1.[88] Additionally, the large percentage of basic residues in positions −5, −4, and −3 may represent activation of basophilic serine/threonine kinases, such as PKCs and Akt kinase, which possess a strong preference for basic residues at position −3.[89,90]

From a different perspective, dataset level analysis may reveal information regarding regulation of kinase activation through increased (or decreased) levels of various kinase motifs in response to diverse stimuli (Figure 4b). Based on high stringency (which means that the site scores in the upper 0.2% of all sites in SwissProt) analysis of our data set with Pattern Explorer, motifs for basophilic serine/threonine kinase group including Akt kinase, PKA, PKCs comprise the largest number of sites (12.9%) followed by motifs for acidophilic (6.9%) and

proline-dependent serine/threonine kinases (5.2%). These patterns not only implicate endogenously activated kinases in HT-29 colon adenocarcinoma cells, but also demonstrate the potential usage of Pattern Explorer for detecting altered kinase activities in response to diverse stimuli such as growth factors and kinase inhibitors. It is important to note that high stringency analysis of the data results in many (74%) phosphorylation sites returned without potential kinase motif or binding group hits. This result is not surprising considering that the human kinome contains 518 putative protein kinases[91] while the Scansite algorithm uses only 26 kinases. As more kinase motifs are identified, it should be possible to extract even more information from large protein phosphorylation data sets.

At the individual site level, kinases matching the motif presented by each peptide phosphorylation site are still easily accessible. Utilization of this interface enables more rapid interrogation of large data sets and facilitates the creation of additional hypotheses regarding the potential function of individual phosphorylation sites. Of course, these hypotheses will still need to be validated by thorough investigation of the literature and additional biological experimentation.

As demonstrated in this work, further development of IMAC−LC−MS/MS has facilitated the rapid collection of a large amount of phosphoproteomic data for the HT-29 cell line. Data generated in this experiment contains a considerable number of phosphorylated peptides from a smaller number of HT-29 cells (1×10^6 cell equivalents) as compared to previous large-scale protein phosphorylation studies. We have identified well-characterized phosphorylation sites as well as many sites that have not been previously reported in the scientific literature. In sorting through the data, we have attempted to present examples of protein phosphorylation sites on key regulatory proteins identified in our results. Quantification of protein phosphorylation at these sites should provide important information in elucidating signaling pathways in HT-29 exposed to diverse cellular signals.

Conclusions

In total, 238 phosphorylation sites (including 25 sites for which we could not conclusively localize phosphorylation) from 116 proteins were identified from IMAC LC/MS/MS analysis of esterified, tryptic peptides from HT-29 human colon adenocarcinoma cells. Although the majority of the sites identified in this effort were from abundant proteins associated with structural maintenance or basic cellular biochemistry, phosphorylation sites were also found on apoptosis-associated proteins and on several proteins associated with regulation of cellular signal transduction, including kinases and their substrates. This later group of phosphorylation sites from signaling proteins identified in our experiment would be good target candidates for quantitative analysis to map signaling pathways in HT-29 in response to diverse stimuli such as pro-apoptotic and anti-apoptotic signals.

Abbreviations: IMAC, immobilized metal-affinity chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; ESI-Q-TOF, electrospray ionization-quadrupole-time-of-flight.

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Supporting Information Available: The entire dataset of protein phosphorylation sites identified in this work (Supplementary Table 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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