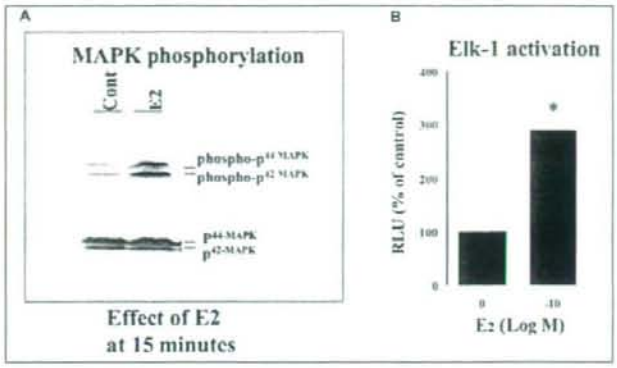

Figure 4.

A) Effect of 0.1 nM estradiol on levels of activated and total MAP kinase measured 15 min after addition of steroid. Shown on the top segment is activated MAP kinase as assessed by an antibody specific for activated MAP kinase and on the bottom segment, total MAP kinase. B) Effect of 0.1 nM estradiol on the activation of ELK-1.

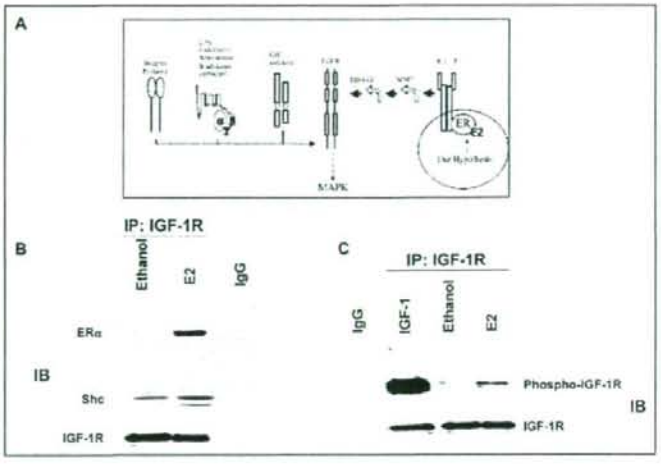

Figure 5.

A, top) Diagrammatic representation of a model in which estradiol binds to ERα which then binds to the adaptor protein, SHC. At the same time estradiol causes phosphorylation of the IGF-1-R, which provides a binding site for SHC. In this model, estradiol signals through the IGF-1-R and activates MAP kinase which then acts through Elk-1 to initiate gene transcription. B) estradiol-induced protein complex formation among ERα, SHC and IGF-1-R. MCF-7 cells were treated with vehicle, 1 ng/ml IGF-1, or E_2 at 0.1 nM for the times indicated. Lysates were immunoprecipitated with IGF-1-R antibody. The nonspecific monoclonal antibody (IgG) served as a negative control.[28] C) estradiol increases the phosphorylation of the IGF-1-R.

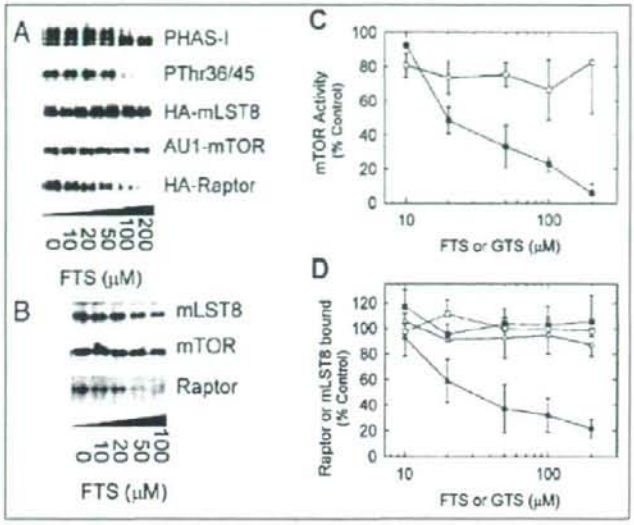

Figure 6.

Left) FTS promotes raptor dissociation and inhibits mTOR activity in cell extracts. A) 293T cells were transfected with pcDNA3 alone (vector) or with a combination of pcDNA3-AU---mTOR, pcDNA3-3-HA-raptor and pcDNA3-3HA-mLST8. Extracts of cells were incubated with increasing concentration of FTS for 30 min before AU-1-mTOR was immunoprecipitated. Samples of the immune complexes were incubated with (γ32P)-ATP and recombinant (HIS 6) PHAS-1 and then subjected to SDS-PAGE. A phosphor image of a dried gel was obtained to detect ^{32}P-PHAS1 and an immunoblot was prepared with PThr36/45 antibodies. Other samples of the immune complexes were subjected to SDS-PAGE and immunoblots were prepared with antibodies to the HA epitope or to mTOR.[2] B) Extracts of nontransfected 293T cells were incubated with increasing concentrations of FTS before mTOR was immunoprecipitated with mTab 1. A control immunoprecipitation was conducted using nonimmune IgG(Nl). Immune complexes were subjected to SDS-PAGE and immunoblots were prepared with antibodies to mLST8, mTOR and raptor.[2] Right) Relative effects of increasing concentrations of FTS and GTS on mTOR activity and the association of mTOR and raptor. Samples of extracts from 293T cells overexpressing AU1-mTOR, HA-raptor and HA-mLST8 were incubated for 1 hr with increasing concentrations of FTS (●, ◆, ■) or GTS (○, Δ, □) before immunopreciptations were conducted with anti-AU 1 antibodies.[2] A) mTOR kinase activity (●, ○) was determined by measuring^{32}P incorporation into (HIS6) PHAS-1 in immune complex kinase assays performed with (γ32P)-ATP. B) The relative amounts of HA-raptor (◆,○) and HA-mLST8 (■, Δ) that co-immunoprecipitated with AU-1-mTOR were determined after immunoblotting with anti-HA antibodies. The results (mean values ± SE for five experiments) are expressed as percentages of the mTOR activity (C) or co-immununoprecipitating proteins (D) from samples incubated without FTS or GTS and have been corrected for the amounts of AU-1–mTOR immunopecipitated.[2] From: McMahon LP et al. J Mol Endocrinol 2005; 19(1):175–183;[2] with permission of The Endocrine Society.

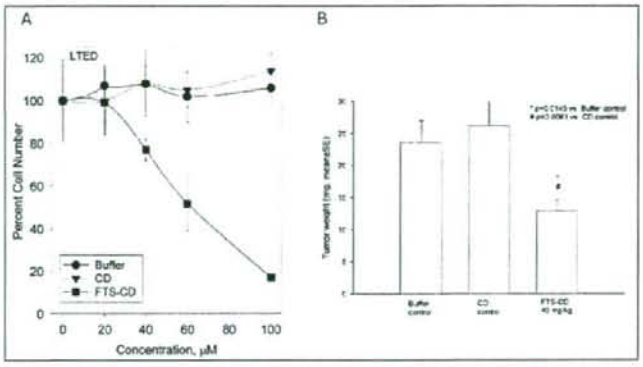

Figure 7.

A) In vitro effects of FTS on cell growth. Effects of FTS complexed with cyclodextrin (CD) for solubility were compared with buffer or cyclodextrin (CD) alone on the number of LTED cells expressed as a percent of maximum number. The ordinate shows the concentration of FTS used. B) In vivo effects of FTS on cell growth. LTED cells were implanted into castrate nude mice to form xenografts. Silastic implants delivering estradiol at amounts sufficient to provide plasma levels of estradiol of 5 pg/ml were implanted. One group received buffer alone, the second cyclodextrin alone and the third FTS 40 mg/kg complexed to cyclodextrin. The effects of FTS-CD compared to CD control were statistically significant at $p = 0.0061$.

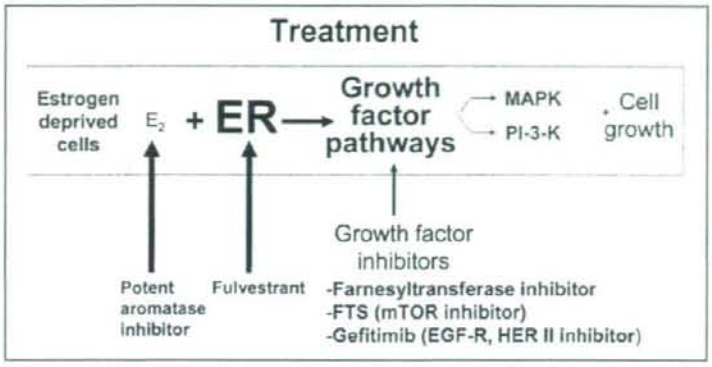

Figure 8.
Practical implications of the effects of up-regulation of growth factor pathways and development of hypersensitivity to estradiol. Potent aromatase inhibitors are useful to counteract the enhanced sensitivity to estradiol resulting from adaptation to prolonged estradiol deprivation. A pure antiestrogen such as fulvestrant can counteract the up-regulation of the ER that occurs. Growth factor inhibitors such as FTS, farnesyl-transferase inhibitors and growth factor inhibitors such as Iressa and others can be used to block up-regulation of growth factor pathways.

technical notes Journal of proteome research

Successive and Selective Release of Phosphorylated Peptides Captured by Hydroxy Acid-Modified Metal Oxide Chromatography

Yutaka Kyono,[†,‡] Naoyuki Sugiyama,[†,§] Koshi Imami,[†] Masaru Tomita,[†,§] and Yasushi Ishihama*[,†,∥]

Institute for Advanced Biosciences, Keio University, Tsuruoka, Yamagata 997-0017, Japan, GL Sciences, Inc., 237-2 Sayamagahara, Iruma, Saitama 358-0032, Japan, Human Metabolome Technologies, Inc., Kakuganji, Tsuruoka, Yamagata 997-0052, Japan, and PRESTO, Japan Science and Technology Agency, Sanbancho Bldg., 5-Sanbancho, Chiyodaku, Tokyo 102-0075, Japan

Received April 22, 2008

Abstract: We developed a novel approach to enlarge phosphoproteome coverage using successive elution of phosphopeptides with various buffers in series from a single microcolumn packed with hydroxy acid-modified metal oxides, such as titania and zirconia. Elution conditions were investigated to maximize the recovery of phosphopeptides from three standard phosphoproteins. Secondary amines, such as piperidine and pyrrolidine, provided better efficiency than the conventional conditions using ammonium hydroxide and phosphate buffers. Furthermore, elution with these secondary amines provided unique phosphopeptides that were not eluted under the conventional conditions in the analysis of HeLa cell lysates. On the basis of these results, we fractionated phosphopeptides captured by a single metal oxide microcolumn using successive elution with 5% ammonium hydroxide solution, 5% piperidine solution and 5% pyrrolidine solution in series. We identified 1803 nonredundant phosphopeptides from 100 μg of HeLa cells, which represented a 1.6-fold increase in phosphopeptide number and a 1.9-fold increase in total peak area of phosphopeptides in comparison with the results obtained under the conventional conditions. Since this approach is applicable to any single tip-based protocol without coupling with other enrichment methods, this simple strategy can be easily incorporated as an option into existing protocols for phosphopeptide enrichment, and would be suitable for high-throughput analysis in a parallel format.

Keywords: phosphoproteome • hydroxy acid modified-metal oxide chromatography • phosphopeptide • pyrrolidine • piperidine • titania • zirconia

Introduction

Reversible phosphorylation of serine, threonine and tyrosine regulates a variety of biological processes, such as intercellular communication, cell growth, proliferation, differentiation, and apoptosis.[1,2] Characterization of phosphorylation status is therefore crucial for the elucidation of signal transduction pathways, as well as for an understanding of the mechanisms of disease and drug actions.

Recent advances in mass spectrometry-based proteomics coupled with phosphopeptide enrichment methods allow the simultaneous identification of thousands of phosphorylation sites.[3−5] The most widely used approaches for phosphopeptide enrichment are immobilized metal affinity chromatography (IMAC)[6−8] and metal oxide chromatography (MOC).[9−16] In both cases, however, nonphosphorylated peptides containing multiple acidic residues bind to the chromatographic beads, disturbing the identification of phosphopeptides.[7]

Neutralizing the carboxyl groups by *O*-methyl esterification has been shown to enhance the specificity of phosphopeptide binding in IMAC,[17,18] while optimized pH together with optimized organic solvent content provided improved results without O-methylation in IMAC.[8] Alternatively, significant improvement was obtained in MOC with titania when benzoic acid derivatives such as 2,5-dihydroxybenzoic acid (DHB) and phthalic acid were used in the sample loading buffer to eliminate acidic nonphosphopeptides.[19] Although these methods provide wider coverage of the phosphoproteome in combination with prefractionation based on SCX, SDS-PAGE and protein IMAC, it is necessary to combine two or more phosphopeptide enrichment methods for evaluation of proteome-wide phosphorylation, as different phosphopeptide enrichment methods are likely to have distinct preferences for particular properties of phosphopeptides.[20,21]

Recently, we found that aliphatic hydroxy acids such as lactic acid and 3-hydroxypropanoic acid work more efficiently and more specifically than aromatic modifiers such as DHB and phthalic acid in titania and zirconia MOC.[22] In this aliphatic hydroxy acid-modified MOC (HAMMOC) mode, no prefractionation was necessary, and more than 1000 unique phosphorylation sites were identified in highly complex cellular extracts from less than 100 μg of nonstimulated HeLa cells.[23] This observation was recently confirmed by another group.[24] We also modified the physicochemical properties of titania beads by calcination at 800 °C and the resultant rutile-form titania showed a completely different preference for multiply phosphorylated and hydrophobic peptides.[25]

* To whom correspondence should be addressed. Yasushi Ishihama, E-mail: y-ishi@ttck.keio.ac.jp; phone, +81-235-29-0571; fax, +81-235-29-0536.
† Keio University.
‡ GL Sciences, Inc.
§ Human Metabolome Technologies, Inc.
∥ PRESTO.

10.1021/pr800305y CCC: $40.75

Published on Web 09/04/2008

Table 1. Identification and Quantification of Phosphopeptides Captured by HAMMOC with Different Elution Conditions[a]

				no. of Identified Peptides (avg.)		relative peak area (%)		
resin	elution buffer	conc. (%)	pH	phosphopeptide	nonphosphopeptide	phosphopeptide	nonphosphopeptide	ratio (%)
Titania	ammonium hydroxide	0.5	11.4	9	1	100.0	0.7	99.3
		5.0	12.1	15	0	151.9	0.8	99.5
		10.0	12.6	12	2	101.4	4.8	95.5
	piperidine	0.5	11.9	14	4	120.3	6.0	95.2
		5.0	12.5	14	3	171.7	2.8	98.4
		10.0	12.8	17	3	139.0	6.3	95.6
	pyrrolidine	0.5	12.1	14	2	157.1	1.4	99.1
		5.0	12.9	14	2	182.3	2.7	98.5
		10.0	13.0	17	2	90.6	3.7	96.1
	piperazine	0.5	11.3	13	1	137.3	1.7	98.8
	methylamine	0.5	12.0	10	1	114.1	1.4	98.8
	dimethyamine	0.5	11.9	12	1	122.4	1.2	99.0
	trimethylamine	0.5	11.4	7	0	79.4	0.4	99.5
	triethylamine	0.5	11.9	14	3	92.0	4.7	95.2
	sodium hydroxide	0.5	12.1	4	0	20.7	0.0	100.0
	disodium hydrogenphosphate	5.0	11.4	5	0	22.9	0.0	100.0
Zirconia	ammonium hydroxide	0.5	11.4	9	3	100.0	0.7	99.3
		5.0	12.1	14	2	126.3	1.2	99.1
		10.0	12.6	17	8	108.2	21.5	83.4
	piperidine	0.5	11.9	13	3	159.4	2.1	98.7
		5.0	12.5	16	1	160.0	1.1	99.3
		10.0	12.8	17	5	102.2	6.6	93.9
	pyrrolidine	0.5	12.1	8	1	130.4	0.7	99.4
		5.0	12.9	14	2	225.2	3.9	98.3
		10.0	13.0	14	6	86.7	7.6	91.9
	piperazine	0.5	11.3	8	2	105.6	1.0	99.1
	methylamine	0.5	12.0	11	0	141.6	0.1	100.0
	dimethyamine	0.5	11.9	13	0	159.7	0.0	100.0
	trimethylamine	0.5	11.4	8	1	85.5	0.5	99.4
	triethylamine	0.5	11.9	13	5	96.4	10.6	90.1
	sodium hydroxide	0.5	12.1	10	0	65.4	0.0	100.0
	disodium hydrogenphosphate	5.0	11.4	7	0	34.2	0.0	100.0

[a] Tryptic digests of α-casein, fetuin and phosvitin (2.5 μg each) were purified using titania and zirconia HAMMOC (triplicate analyses for each condition). The eluted fractions were analyzed by nanoLC-MS/MS using QSTAR. The total peak area obtained by 0.5% ammonium hydroxide elution was used as a control and the relative peak areas were calculated for other elution conditions.

During the above studies aimed at optimizing HAMMOC, we found that titania and zirconia have, in general, stronger affinity for phosphopeptides than does IMAC, and few phosphopeptides were observed in the flow-through fractions, but the recoveries of phosphopeptides varied from a few percent to 100%.[22] It appears that phosphopeptides remain captured in metal oxide resins even after the elution steps.

A variety of reagents have been used to release phosphopeptides trapped by titania, such as sodium borate with sodium chloride;[9] potassium phosphate;[10] ammonium bicarbonate;[11,19,26] ammonium bicarbonate with acetonitrile;[26] ammonium hydroxide;[19,26] potassium phosphate with ammonium hydroxide;[27] the combination of ammonium bicarbonate, phosphate, orthovanadate, and fluoride;[28,29] and triethylammonium bicarbonate followed by ammonium hydroxide.[30] However, only a limited number of phosphopeptides from standard phosphoproteins have been examined under these elution conditions, and to our knowledge, no group has compared elution conditions using complex samples such as cell lysates. It would, therefore, be difficult to extrapolate the elution conditions based on these small-scale experiments to large-scale phosphoproteome studies.

In this study, we examined various kinds of elution buffers in HAMMOC using phosphopeptides from not only standard phosphoproteins, but also complex cell lysates. On the basis of the results, we developed an approach based on successive and selective elution to enlarge the phosphoproteome coverage by HAMMOC. In addition, we proposed a characterization method for elution selectivity, using seven descriptors based on the physicochemical properties of phosphopeptides identified by each enrichment method.

Experimental Procedures

Materials. Titania (titanium dioxide, particle size: 10 μm) was obtained from GL Sciences (Tokyo, Japan). Zirconia (zirconium dioxide, particle size: 10 μm) was from ZirChrom Separations (Anoka, MN). C2 and C18 Empore disks were from 3 M (St. Paul, MN). Bovine α-casein, bovine fetuin, chicken phosvitin, and PHOS-Select Iron affinity gel were from Sigma (St. Louis, MO). 3-Hydroxypropanoic acid (HPA) was obtained from Tokyo Kasei (Tokyo, Japan). Modified trypsin was from Promega (Madison, WI). MS-grade Lys-C, DL-lactic acid, and all other chemicals were purchased from Wako (Osaka, Japan). Water was obtained from a Millipore Milli-Q system (Bedford, MA).

Preparation of Standard Phosphoprotein Mixtures. α-Casein, fetuin, and phosvitin were individually reduced with dithiothreitol (DTT), alkylated with iodoacetamide, and digested with Lys-C, followed by dilution and trypsin digestion, as described.[31] These digested samples were desalted using StageTips with C18 Empore disk membranes.[32] The eluates were mixed and the concentration was adjusted to 0.5 mg/mL for each protein with 0.1% TFA and 80% acetonitrile.

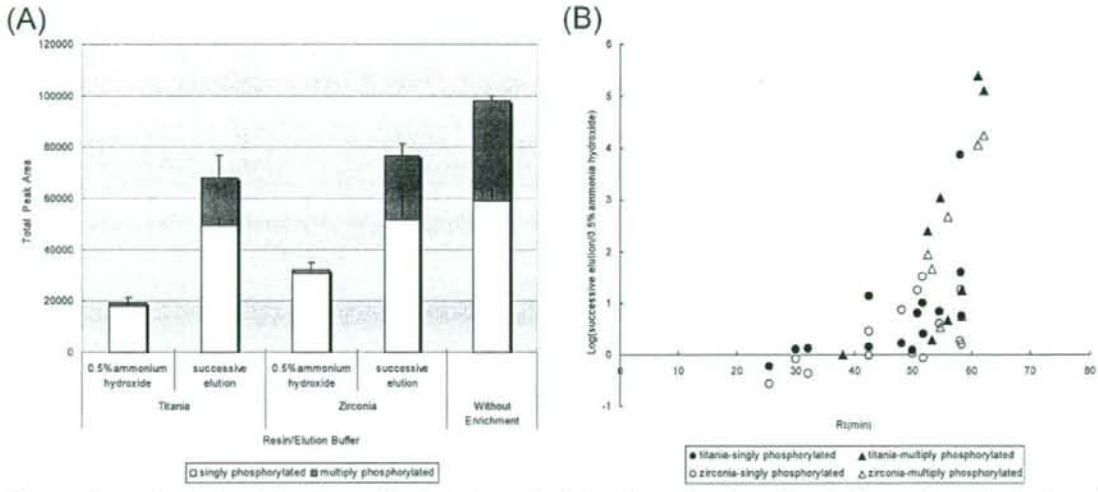

Figure 1. Successive elution of phosphopeptides from the standard phosphoprotein mixture. Tryptic digests of α-casein, fetuin and phosvitin (2.5 μg each) were purified using lactic acid-modified titania and HPA-modified zirconia HAMMOC tips. The bound phosphopeptides in HAMMOC tips were released by successive elution with three buffers, 5% ammonium hydroxide buffer, 5% piperidine buffer, and 5% pyrrolidine buffer, in series. (A) Total peak area of identified phosphopeptides. Averaged values based on triplicate analyses were used. (B) Dependence of peak area ratio between 0.5% ammonium hydroxide elution and the successive elution on the retention times in LC-MS analysis for 22 phosphopeptides identified by titania and zirconia HAMMOC. Closed circle, singly phosphorylated peptides by titania HAMMOC; closed triangle, multiply phosphorylated peptides by titania HAMMOC; open circle, singly phosphorylated peptides by zirconia HAMMOC; open triangle, multiply phosphorylated peptides by zirconia HAMMOC.

Preparation of HeLa Cell Cytoplasmic Fractions. HeLa cells were prepared as described previously.[22] The cell pellet was dissolved in 100 mM Tris buffer (pH 8.0), and protein phosphatase inhibitor cocktails 1 and 2 (Sigma) and protease inhibitors (Sigma) were added. After homogenization with a Dounce homogenizer (10 strokes), the resultant solution was centrifuged at 1500*g* for 10 min, and the supernatant was digested and desalted as shown above. The total protein concentration was adjusted to 1.0 mg/mL with 0.1% TFA and 80% acetonitrile.

Enrichment of Phosphopeptides from Standard Phosphoprotein Digests Using HAMMOC Tips. Phosphopeptide enrichment was performed according to the protocol described previously,[22] except for the elution conditions. Briefly, C2-StageTips packed with metal oxide bulk beads (1 mg beads/10 μL pipet tip) were equilibrated with 0.1% TFA and 80% acetonitrile including aliphatic hydroxy acids as selectivity chelating enhancers (solution A). As the chelating enhancers, lactic acid at the concentration of 300 mg/mL and HPA at 100 mg/mL were used for tips with titania and zirconia, respectively. A 15 μL aliquot of the digested standard phosphoprotein solution was mixed with solution A, and loaded onto the HAMMOC tip, followed by successive washing with solution A and solution B (0.1% TFA in 80% acetonitrile). Various amines and other salts, such as ammonium hydroxide, methylamine, dimethylamine, trimethylamine, pyrrolidine, piperidine, piperazine, sodium hydroxide, and disodium hydrogen phosphate, at different pH values and concentrations were employed to elute the bound phosphopeptides. In the case of multiple elution, three elution solutions (5% ammonium hydroxide, 5% piperidine, and 5% pyrrolidine) were used successively in this order. Note that no organic solvent was added to these elution buffers. The fractions were immediately acidified, and were desalted using C18 StageTips.[23] A Tomy CC-105 vacuum evaporator (Tokyo, Japan) was used to concentrate the sample. After dissolving the phosphopeptides with solution A, nanoLC-MS/MS analysis was performed.

Immobilized Metal Affinity Chromatography (IMAC). IMAC was performed according to the procedure described by Kokubu et al.,[8] with some modifications as follows. PHOS-Select gel (IMAC-Fe^{3+} type, 30 μL) was loaded onto a 200 μL pipet tip packed with C2 Empore disk instead of C18 disk. Sample solutions in 0.1%TFA and 80% acetonitrile were diluted 5 times with Milli-Q water and then loaded onto the IMAC/C2 tips. The tips were washed with 0.3% TFA; then, 50% acetonitrile and 0.5% ammonium hydroxide were used for eluting bound phosphopeptides. The fractions were worked up for nanoLC-MS/MS analysis as described above.

NanoLC-MS System. NanoLC-MS/MS analyses were conducted using a QSTAR XL mass spectrometer (AB/MDS-Sciex, Toronto, Canada) equipped with an Agilent 1100 nanoflow pump (Waldbron, Germany) and an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland), or an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, Bremen, Germany) equipped with a Dionex Ultimate3000 pump (Germering, Germany) and the HTC-PAL autosampler. ReproSil C18 materials (3 μm, Dr. Maisch, Ammerbuch, Germany) were packed into a self-pulled needle (150 mm length × 100 μm i.d., 6 μm opening) with a nitrogen-pressurized column loader cell (Nikkyo Technos, Tokyo, Japan) to prepare an analytical column needle with "stone-arch" frit.[33] The injection volume was 5 μL and the flow rate was 500 nL/min. The mobile phases consisted of (A) 0.5% acetic acid and (B) 0.5% acetic acid in 80% acetonitrile. Two gradient conditions were employed, that is, 5−30% B in 20 min, 30−100% B in 5 min and 100% B for 10 min for the standard phosphoprotein digests, and 5−10% B in 5 min, 10−40% B in 60 min, 40−100% B in 5 min and 100% B

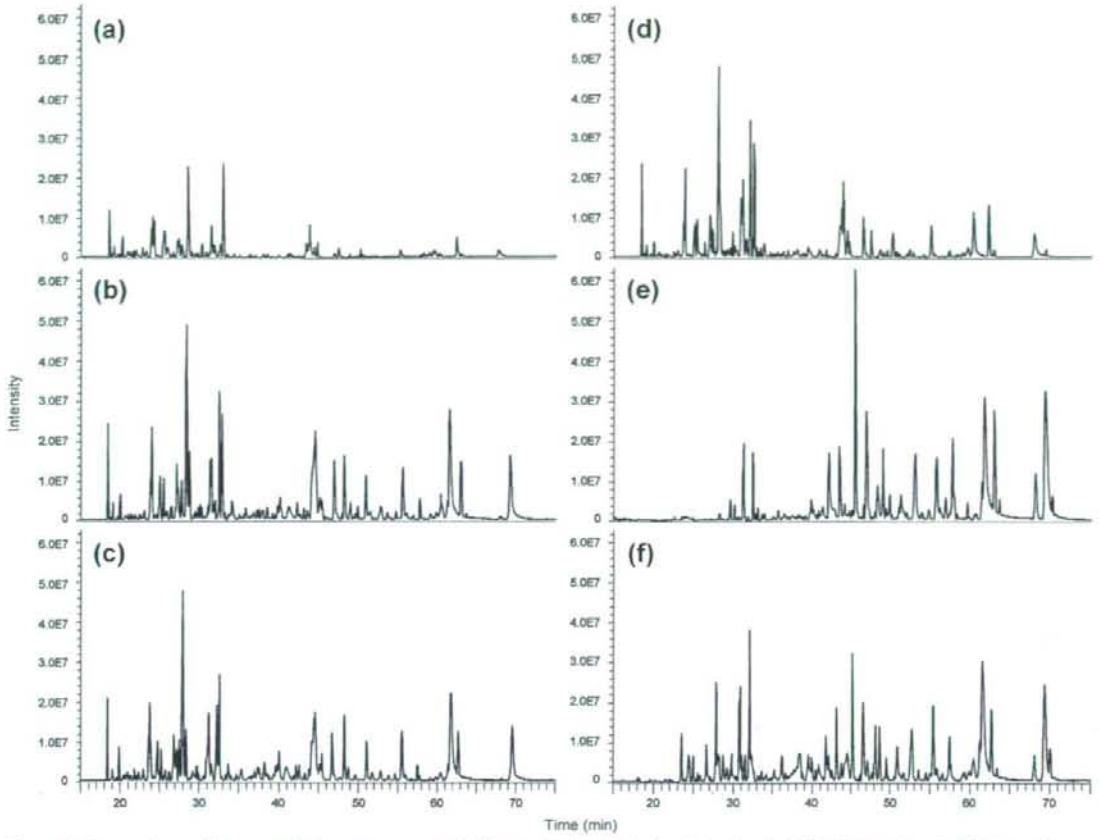

Figure 2. Comparison of base peak chromatograms of HeLa cell extract, obtained by titania-HAMMOC under six different elution conditions. Elution with (a) 0.5% ammonium hydroxide, (b) 0.5% piperidine, (c) 0.5% pyrrolidine, (d) 5% ammonium hydroxide, (e) 5% piperidine, and (f) 5% pyrrolidine. The elution procedures as well as LC-MS conditions are described in the Experimental Procedures.

Table 2. Overlap Analysis between Phosphopeptides in HeLa Cell Lysates Obtained by Titania HAMMOC with Six Different Elution Conditions[a]

	0.5% ammonium hydroxide	0.5% piperidine	0.5% pyrrolidine	5% ammonium hydroxide	5% piperidine	5% pyrrolidine
0.5% ammonium hydroxide	100	38.4	39.0	43.5	10.8	21.0
0.5% piperidine	38.4	100	48.8	49.0	23.9	35.9
0.5% pyrrolidine	39.0	48.8	100	48.0	17.0	29.8
5% ammonium hydroxide	43.5	49.0	48.0	100	19.1	30.0
5% piperidine	10.8	23.9	17.0	19.1	100	36.1
5% pyrrolidine	21.0	35.9	29.8	30.0	36.1	100

42-100%
36-42%
30-36%
0-30%

[a] The overlap percentages between systems A and B were calculated using the following equation: Overlap(%) = $(A \cap B/A \cup B) \times 100$.

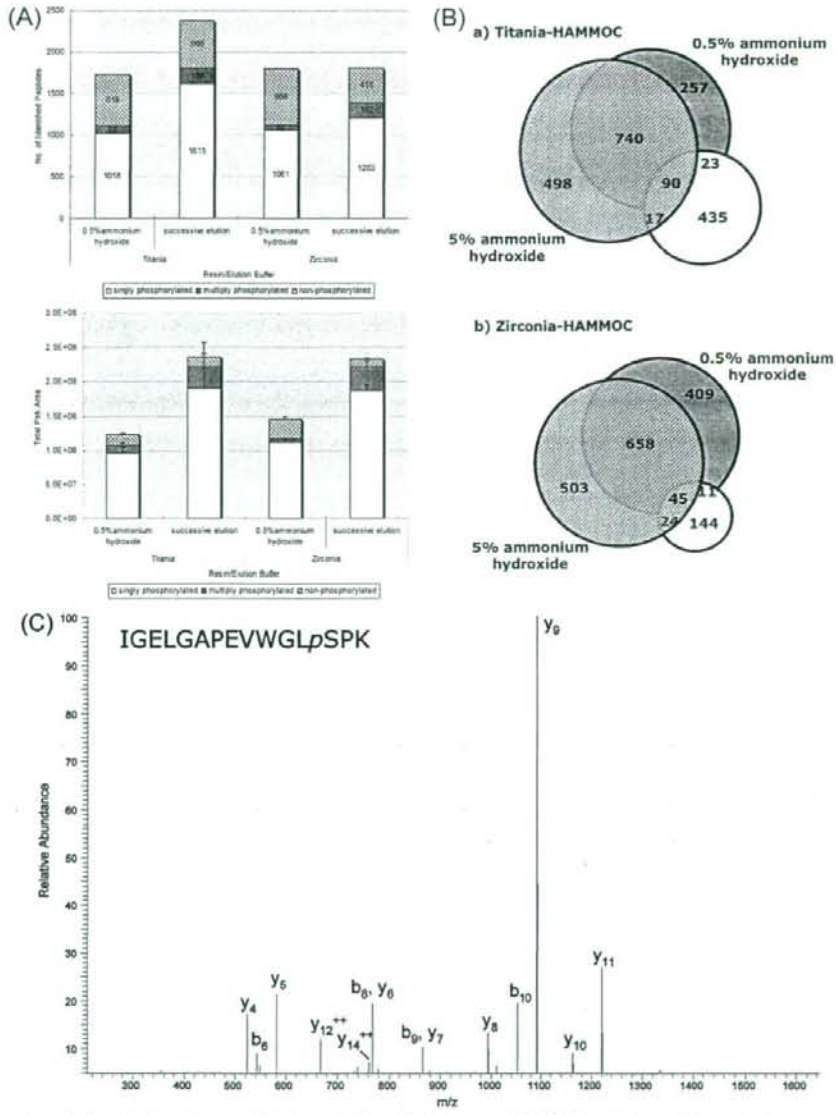

Figure 3. Successive elution of phosphopeptides from HeLa cell lysates in HAMMOC. (A) Comparison of the number of identified peptides and total peak area upon 0.5% ammonium hydroxide elution with that upon successive elution in titania and zirconia HAMMOC. Triplicate analyses were carried out and the merged results were described for the peptide number, whereas averaged values were employed for the peak area. (B) Venn diagrams showing the numbers of identified phosphopeptides obtained by 0.5% ammonium hydroxide elution and the successive elution. The eluates were separately collected and analyzed by LC-MS in the successive elution mode. (C) Example of an MS/MS spectrum assigned to IGELGAPEVWGLpSPK from NF-kappa-B-activating protein (NKAP_HUMAN). The precursor ion (*m/z* 816.903539, 2+) was isolated at 57.7 min and fragmented by collision-induced dissociation (CID) in the linear ion trap. Fragment ions containing the N-(*b-type* ions) or C-(*y-type* ions) terminus are labeled. The ion score of 70 was obtained for this spectrum.

for 10 min for the HeLa lysates. A spray voltage of 2400 V was applied. The MS scan range was *m/z* 350−1400 (QSTAR) or 300−1500 (LTQ-Orbitrap). For QSTAR experiments, MS scans were performed for 1 s to select three intense peaks, and subsequently three MS/MS scans were performed for 0.55 s each. An information-dependent acquisition function was activated for 2 min to exclude the previously scanned parent ions. The CID energy was automatically adjusted by rolling CID function. For LTQ-Orbitrap, the top 10 precursor ions were selected in MS scan by Orbitrap with $R = 60\,000$ for subsequent MS/MS scans by ion trap in the automated gain control (AGC) mode where AGC values of 5.00×10^5 and 1.00×10^4 were set for full MS and MS/MS, respectively. The normalized CID was set to be 35.0.

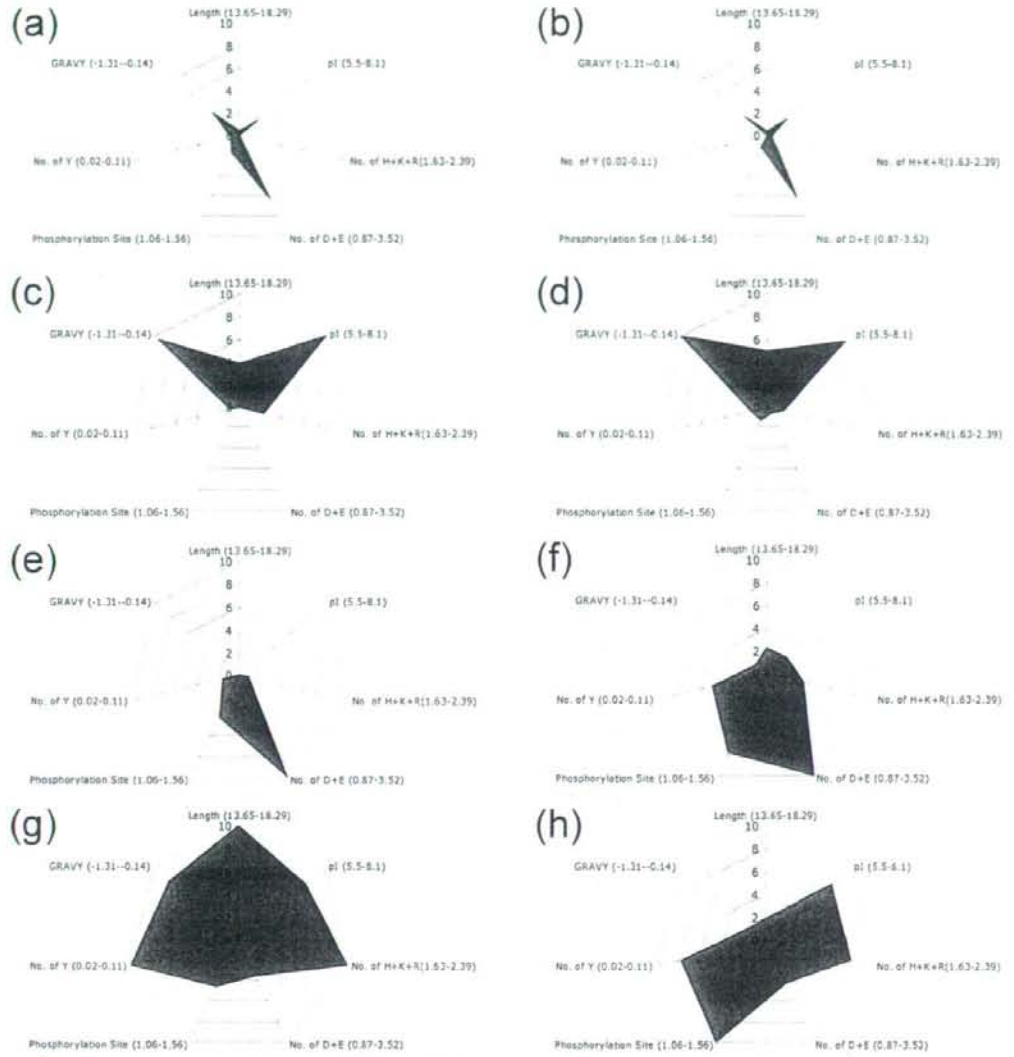

Figure 4. Heptagonal radar graphs for characterization of eight different enrichment methods using phosphopeptides exclusively obtained by each method: (a) titania with ammonia hydroxide elution, (b) zirconia with ammonium hydroxide elution, (c) titania with piperidine and pyrrolidine elution, (d) zirconia with piperidine and pyrrolidine elution, (e) titania with phosphate elution, (f) zirconia with phosphate elution, (g) rutile titania with ammonium hydroxide elution, and (h) IMAC with ammonium hydroxide elution. The descriptors are the length of peptide expressed in terms of the number of amino acid residues (length), the isoelectric points of peptides (p*I*), the total number of histidine, lysine, and arginine residues per peptide (No. of H+K+R), the number of aspartic acid and glutamic acid residues per peptide (No. of D+E), the averaged number of phosphorylation sites per peptide (phosphorylation site), the averaged number of phosphotyrosine residues per peptide (No. of Y) and the GRAVY score[37] indicating the hydrophobicity of peptides (GRAVY). The maximum and minimum values of each descriptor are shown on each axis. The data set used for this calculation corresponds to the shaded parts of the Venn diagrams shown in Supplemental Figure 5 in Supporting Information.

Database Searching. Mass Navigator v1.2 (Mitsui Knowledge Industry, Tokyo, Japan) was used to create peak lists on the basis of the recorded fragmentation spectra. Peptides and proteins were identified by means of automated database searching using Mascot v2.1 (Matrix Science, London) against UniProt/Swiss-Prot release 54.8 (24-Jan-2008) with a precursor mass tolerance of 0.25 Da (QSTAR) or 3 ppm (LTQ-Orbitrap), a fragment ion mass tolerance of 0.25 Da (QSTAR) or 0.8 Da (LTQ-Orbitrap) and strict trypsin specificity[34] allowing for up to 2 missed cleavages. Cysteine carbamidomethylation was set as a fixed modification, and methionine oxidation and phosphorylation of serine, threonine, and tyrosine were allowed as variable modifications.

Peptides were considered identified if the Mascot score was over the 95% confidence limit based on the 'identity' score of each peptide and at least three successive y- or b-ions with

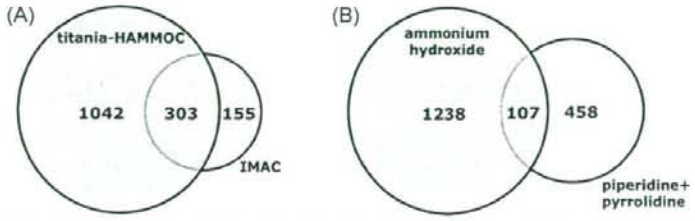

Figure 5. The numbers of identified phosphopeptides obtained (A) by the combination of IMAC with titania-HAMMOC and (B) by the successive elution method with titania HAMMOC. HeLa cell lysates (100 μg) were examined with each method (IMAC, titania HAMMOC with 5% ammonium hydroxide, and titania HAMMOC with successive elution). Triplicate analyses was performed for each method and the obtained results were merged.

two and more y-, b- and/or precursor-origin neutral loss ions were observed, based on an error-tolerant peptide sequence tag concept.[35] A randomized decoy database created by a Mascot Perl program was employed to estimate the false-positive rate for identified peptides within these criteria. Note that most sulfated peptides can be discriminated from phosphopeptides because of the ultrahigh accuracy of the Orbitrap instrument that we used.[36]

Phosphorylated sites were unambiguously determined when y- or b-ions between which the phosphorylated residue exists were observed in the peak lists of the fragment ions. This step was automatically performed using an in-house Perl script 'PhosPep Analyzer' by comparing the original peak lists with the theoretical m/z values of y- or b-ions from Mascot output files.

The total peak area of phosphopeptides and nonphosphopeptides were calculated using the nonlabel quantitation function of Mass Navigator.

Results and Discussion

First, we investigated the influence of various alkaline buffers (pH 11−13) on the elution of phosphopeptides in the standard phosphoprotein mixture. The results obtained with 0.5% ammonium hydroxide elution, which has been used in our standard protocol[22,23] as well as by others,[19] were used as a control, and the relative peak areas were calculated for other elution buffers. We used 8 different amines: ammonium hydroxide, piperidine, pyrrolidine, piperazine, methylamine, dimethylamine, trimethylamine and triethylamine. The obtained results are summarized in Table 1. Pyrrolidine gave the best results in titania HAMMOC, whereas piperidine and dimethylamine were superior to others in zirconia HAMMOC. Kweon et al. also reported that 0.5% piperidine gave better results than ammonium hydroxide buffers in zirconia chromatography.[15] These results suggested that secondary monoamines generally gave better results than other amines, and no clear pH dependence was observed. To confirm the pH effect, we also examined sodium hydroxide (pH 12.1) and disodium hydrogen phosphate (pH 11.4) and found that elution with both buffers resulted in lower recovery in both titania and zirconia HAMMOC, indicating the pH of the elution buffer is not the primary factor that controls the recovery. Next, we increased the concentration of piperidine, pyrrolidine and ammonium hydroxide. The maximum recovery was obtained with 5% elution buffers; an increase to 10% did not yield further improvement in any of these cases. Regarding multiply phosphorylated peptides, the content generally increased as the recovery increased (Supplemental Figure 1 in Supporting Information). Interestingly, we found that the peak profiles of total ion current (TIC) chromatograms of the eluates with 0.5−10% ammonium hydroxide were similar to those of the eluates with 0.5% piperidine and 0.5% pyrrolidine, while 5% piperidine and pyrrolidine elution gave different peak profiles in TIC from those in the case of 5% ammonium hydroxide elution (Supplemental Figure 2 in Supporting Information). Therefore, to maximize the recovery of each phosphopeptide, we eluted these phosphopeptides using successive loading with the three buffers, 5% ammonium hydroxide, 5% piperidine, and 5% pyrrolidine, in series. The results are shown in Figure 1. Both in titania and zirconia HAMMOC, this successive elution approach significantly improved the recovery, especially for hydrophobic phosphopeptides and multiply phosphorylated peptides. Since this phosphoprotein mixture contains only a small number of phosphopeptides, we applied this approach to the more complex HeLa cell lysate. To address the characteristics of phosphopeptides eluted under each condition, elution buffers such as ammonium hydroxide, piperidine and pyrrolidine buffers at 0.5% and 5% concentrations were individually examined. As observed in the standard phosphoprotein mixture, 5% piperidine and 5% pyrrolidine provided different peak profiles in their base peak chromatograms (BPCs) from other conditions in titania HAMMOC (Figure 2). This was confirmed by overlap analysis as summarized in Table 2; that is, the overlaps between 0.5% ammonium hydroxide, 5% ammonium hydroxide, 0.5% piperidine and 0.5% pyrrolidine were in the range of 38−49%, whereas the overlaps between 5% piperidine and the other four buffers (except 5% pyrrolidine buffer) were in the range of 11−24% and the overlaps between 5% pyrrolidine and the other four buffers (except 5% piperidine buffer) were in the range of 21−36%. These results showed that 5% piperidine and 5% pyrrolidine each gave a unique selectivity in phosphopeptide identification. On the other hand, similar profiles were obtained for all elution buffers in zirconia HAMMOC, though the averaged peak intensities were different (see Supplemental Figure 3 and Supplemental Table 1 in Supporting Information).

To maximize coverage of the phosphoproteome by titania HAMMOC, we examined the fractionation obtained with successive loading of different elution buffers onto a single titania tip. On the basis of the results in Table 2, we selected 5% ammonium hydroxide, 5% piperidine and 5% pyrrolidine for phosphopeptide fractionation. Because the peptide content in the second and third fractions were expected to be relatively smaller than in the first fraction, these fractions were combined. In total, 1803 unique phosphopeptides were successfully identified by this fractionation approach and the gain from use

of the combined fraction was more than 450 peptides in titania HAMMOC, whereas 1385 phosphopeptides were identified in total and only 155 phosphopeptides were uniquely identified in the combined fraction in zirconia HAMMOC (Figure 3, Supplemental Table 2 in Supporting Information) with the false-positive rate of 1.15% for merged LC-MS results. We also confirmed that replicate elution with 5% ammonium hydroxide did not provide a significant gain in the second fractions (Supplemental Figure 4 in Supporting Information), indicating that the elution with 5% piperidine and 5% pyrrolidine provided phosphopeptides that were not eluted with 5% ammonium hydroxide. Although phosphopeptide enrichment approaches reported so far are generally complementary, it is important to understand the difference between each method quantitatively in order to maximize the phosphoproteome coverage with minimum effort. We selected 7 different properties to describe the characteristics of each method, as shown in Figure 4. The heptagonal radar graphs indicate that phosphopeptides uniquely identified by 5% piperidine−5% pyrrolidine elution are more hydrophobic and less acidic in both titania and zirconia HAMMOC than those in the case of 0.5% ammonium hydroxide elution, whereas other properties, including the length of peptides, are almost identical with those in the case of 0.5% ammonium hydroxide elution. Phosphopeptides exclusively eluted with the phosphate buffer have more acidic and more hydrophilic properties. Interestingly, we did not find any distinct difference between titania and zirconia HAMMOC under the three different elution conditions, except that the recoveries were quite different, especially for piperidine−pyrrolidine buffers. Nevertheless, the combination of titania and zirconia HAMMOC resulted in wider coverage than replicate analysis using either HAMMOC alone.[22,23] Similar profiles were observed for the rutile form of titania and IMAC, where more basic, less acidic, and more phosphorylated peptides were observed. The unique features of rutile-titania are that longer and more hydrophobic peptides can be identified. The problem with this system arises from contamination with nonphosphopeptides, although direct coupling with LC-MS systems was possible because no modifiers are needed in rutile-titania chromatography.[25] For IMAC, as reported by other groups,[20,21,27] more phosphorylated peptides were observed and this would seem to be the most suitable system for complementary usage with titania or zirconia HAMMOC. However, the overlap between IMAC and titania-HAMMOC-5% ammonium hydroxide elution was larger than that between titania HAMMOC with 5% ammonium hydroxide and titania HAMMOC with 5% piperidine-5% pyrrolidine (Figure 5). In addition, the gain by IMAC was smaller than that by titania-HAMMOC with 5% piperidine-5% pyrrolidine. Although the unique feature of IMAC is still complementarity to titania-HAMMOC, the first choice to enlarge the extent of phosphoproteome identification was to use another elution condition (5% piperidine-5% pyrrolidine) in titania HAMMOC, rather than the recently reported SIMAC strategy, where IMAC was used as a first filter to capture multiply phosphorylated peptides and the flow-through fraction was loaded onto titania.[21] Since our approach based on successive and selective elution allows flexible addition of other elution buffers with different selectivity, or changing the combination of elution buffers and the elution order, this simple strategy could be easily incorporated into existing protocols for phosphopeptide enrichment as an option, and would be suitable for high-throughput analysis in a parallel format.

Data Availability. All MSMS spectra are available free in Keio Univ. Pepbase peptide database (http://pepbase.iab.keio.ac.jp/phospho/jpr).

Acknowledgment. We thank Sumiko Ohnuma and Yasuyuki Igarashi for their technical assistance. This work was partly supported by research funds from Yamagata Prefecture and Tsuruoka City.

Supporting Information Available: Tables of overlap analysis between phosphopeptides in HeLa cell lysates obtained with zirconia-HAMMOC and lists of identified phosphopeptides by titania and zirconia HAMMOC for HeLa cells. Figures of phosphorylation distribution of phosphopeptides captured by HAMMOC with different elution conditions, comparison of total ion current chromatograms obtained with titania-HAMMOC of the tryptic digests of phosphoprotein mixture under three conditions, comparison of base peak chromatograms obtained by zirconia-HAMMOC of the HeLa cell extract under six different elution conditions, duplicate elution of phosphopeptides from the HeLa cell lysates in HAMMOC with 5% ammonium hydroxide, and the data sets used for calculation of the heptagonal radar graphs. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hunter, T. Signaling-2000 and beyond. *Cell* **2000**, *100* (1), 113–27.
(2) Blume-Jensen, P.; Hunter, T. Oncogenic kinase signalling. *Nature* **2001**, *411* (6835), 355–65.
(3) Beausoleil, S. A.; Jedrychowski, M.; Schwartz, D.; Elias, J. E.; Villen, J.; Li, J.; Cohn, M. A.; Cantley, L. C.; Gygi, S. P. Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (33), 12130–5.
(4) Olsen, J. V.; Blagoev, B.; Gnad, F.; Macek, B.; Kumar, C.; Mortensen, P.; Mann, M. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* **2006**, *127* (3), 635–48.
(5) Chi, A.; Huttenhower, C.; Geer, L. Y.; Coon, J. J.; Syka, J. E.; Bai, D. L.; Shabanowitz, J.; Burke, D. J.; Troyanskaya, O. G.; Hunt, D. F. Analysis of phosphorylation sites on proteins from Saccharomyces cerevisiae by electron transfer dissociation (ETD) mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (7), 2193–8.
(6) Stensballe, A.; Andersen, S.; Jensen, O. N. Characterization of phosphoproteins from electrophoretic gels by nanoscale Fe(III) affinity chromatography with off-line mass spectrometry analysis. *Proteomics* **2001**, *1* (2), 207–22.
(7) Haydon, C. E.; Eyers, P. A.; Aveline-Wolf, L. D.; Resing, K. A.; Maller, J. L.; Ahn, N. G. Identification of novel phosphorylation sites on Xenopus laevis Aurora A and analysis of phosphopeptide enrichment by immobilized metal-affinity chromatography. *Mol. Cell. Proteomics* **2003**, *2* (10), 1055–67.
(8) Kokubu, M.; Ishihama, Y.; Sato, T.; Nagasu, T.; Oda, Y. Specificity of immobilized metal affinity-based IMAC/C18 tip enrichment of phosphopeptides for protein phosphorylation analysis. *Anal. Chem.* **2005**, *77* (16), 5144–54.
(9) Ikeguchi, Y.; Nakamura, H. Determination of organic phosphates by column-switching high performance anion-exchange chromatography using on-line preconcentration on titania. *Anal. Sci.* **1997**, *13* (6), 479–483.
(10) Kuroda, I.; Shintani, Y.; Motokawa, M.; Abe, S.; Furuno, M. Phosphopeptide-selective column-switching RP-HPLC with a titania precolumn. *Anal. Sci.* **2004**, *20* (9), 1313–9.
(11) Pinkse, M. W.; Uitto, P. M.; Hilhorst, M. J.; Ooms, B.; Heck, A. J. Selective isolation at the femtomole level of phosphopeptides from proteolytic digests using 2D-NanoLC-ESI-MS/MS and titanium oxide precolumns. *Anal. Chem.* **2004**, *76* (14), 3935–43.
(12) Sano, A.; Nakamura, H. Chemo-affinity of titania for the column-switching HPLC analysis of phosphopeptides. *Anal. Sci.* **2004**, *20* (3), 565–6.
(13) Sano, A.; Nakamura, H. Titania as a chemo-affinity support for the column-switching HPLC analysis of phosphopeptides: application to the characterization of phosphorylation sites in proteins by combination with protease digestion and electrospray ionization mass spectrometry. *Anal. Sci.* **2004**, *20* (5), 861–4.
(14) Wolschin, F.; Wienkoop, S.; Weckwerth, W. Enrichment of phosphorylated proteins and peptides from complex mixtures using

metal oxide/hydroxide affinity chromatography (MOAC). *Proteomics* **2005**, *5* (17), 4389–97.
(15) Kweon, H. K.; Hakansson, K. Selective zirconium dioxide-based enrichment of phosphorylated peptides for mass spectrometric analysis. *Anal. Chem.* **2006**, *78* (6), 1743–9.
(16) Ishihama, Y.; Mann, M. Development of nanoLC-MS/MS systems for proteomics. *Chromatography* **2003**, *24* (Suppl.1), 12–3.
(17) Ficarro, S. B.; McCleland, M. L.; Stukenberg, P. T.; Burke, D. J.; Ross, M. M.; Shabanowitz, J.; Hunt, D. F.; White, F. M. Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae. *Nat. Biotechnol.* **2002**, *20* (3), 301–5.
(18) Ndassa, Y. M.; Orsi, C.; Marto, J. A.; Chen, S.; Ross, M. M. Improved immobilized metal affinity chromatography for large-scale phosphoproteomics applications. *J. Proteome Res.* **2006**, *5* (10), 2789–99.
(19) Larsen, M. R.; Thingholm, T. E.; Jensen, O. N.; Roepstorff, P.; Jorgensen, T. J. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol. Cell. Proteomics* **2005**, *4* (7), 873–86.
(20) Bodenmiller, B.; Mueller, L. N.; Mueller, M.; Domon, B.; Aebersold, R. Reproducible isolation of distinct, overlapping segments of the phosphoproteome. *Nat. Methods* **2007**, *4* (3), 231–7.
(21) Thingholm, T. E.; Jensen, O. N.; Robinson, P. J.; Larsen, M. R. SIMAC (Sequential Elution from IMAC), a phosphoproteomic strategy for the rapid separation of monophosphorylated from multiply phosphorylated peptides. *Mol. Cell. Proteomics* **2008**, *7*, 661–71.
(22) Sugiyama, N.; Masuda, T.; Shinoda, K.; Nakamura, A.; Tomita, M.; Ishihama, Y. Phosphopeptide enrichment by aliphatic hydroxy acid-modified metal oxide chromatography for nano-LC-MS/MS in proteomics applications. *Mol. Cell. Proteomics* **2007**, *6* (6), 1103–9.
(23) Rappsilber, J.; Mann, M.; Ishihama, Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat. Protocols* **2007**, *2* (8), 1896–1906.
(24) Jensen, S. S.; Larsen, M. R. Evaluation of the impact of some experimental procedures on different phosphopeptide enrichment techniques. *Rapid Commun. Mass Spectrom.* **2007**, *21* (22), 3635–45.
(25) Imami, K.; Sugiyama, N.; Kyono, Y.; Tomita, M.; Ishihama, Y. Automated phosphoproteome analysis for cultured cancer cells by two-dimensional nanoLC-MS using a calcined titania/C18 biphasic column. *Anal. Sci.* **2008**, *24* (1), 161–6.
(26) Cantin, G. T.; Shock, T. R.; Park, S. K.; Madhani, H. D.; Yates, J. R. 3rd, Optimizing TiO_2-based phosphopeptide enrichment for automated multidimensional liquid chromatography coupled to tandem mass spectrometry. *Anal. Chem.* **2007**, *79* (12), 4666–73.
(27) Wilson-Grady, J. T.; Villen, J.; Gygi, S. P. Phosphoproteome analysis of fission yeast. *J. Proteome Res.* **2008**, *7* (3), 1088–97.
(28) Mohammed, S.; Kraiczek, K.; Pinkse, M. W.; Lemeer, S.; Benschop, J. J.; Heck, A. J. Chip-based enrichment and nanoLC-MS/MS analysis of phosphopeptides from whole lysates. *J. Proteome Res.* **2008**.
(29) Pinkse, M. W.; Mohammed, S.; Gouw, J. W.; van Breukelen, B.; Vos, H. R.; Heck, A. J. Highly robust, automated, and sensitive online TiO_2-based phosphoproteomics applied to study endogenous phosphorylation in *Drosophila melanogaster*. *J. Proteome Res.* **2008**, *7* (2), 687–97.
(30) Simon, E. S.; Young, M.; Chan, A.; Bao, Z. Q.; Andrews, P. C. Improved enrichment strategies for phosphorylated peptides on titanium dioxide using methyl esterification and pH gradient elution. *Anal. Biochem.* **2008**, *377*, 234–42.
(31) Saito, H.; Oda, Y.; Sato, T.; Kuromitsu, J.; Ishihama, Y. Multiplexed two-dimensional liquid chromatography for MALDI and nanoelectrospray ionization mass spectrometry in proteomics. *J. Proteome Res.* **2006**, *5* (7), 1803–7.
(32) Rappsilber, J.; Ishihama, Y.; Mann, M. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* **2003**, *75* (3), 663–70.
(33) Ishihama, Y.; Rappsilber, J.; Andersen, J. S.; Mann, M. Microcolumns with self-assembled particle frits for proteomics. *J. Chromatogr., A* **2002**, *979* (1–2), 233–9.
(34) Olsen, J. V.; Ong, S. E.; Mann, M. Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Mol. Cell. Proteomics* **2004**, *3* (6), 608–14.
(35) Mann, M.; Wilm, M. Error-tolerant identification of peptides in sequence databases by peptide sequence tags. *Anal. Chem.* **1994**, *66* (24), 4390–9.
(36) Bossio, R. E.; Marshall, A. G. Baseline resolution of isobaric phosphorylated and sulfated peptides and nucleotides by electrospray ionization FTICR ms: another step toward mass spectrometry-based proteomics. *Anal. Chem.* **2002**, *74* (7), 1674–9.
(37) Kyte, J.; Doolittle, R. F. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **1982**, *157* (1), 105–32.

PR800305Y

日本薬学会　薬学研究ビジョン部会

Pharma VISION NEWS

No. 12
（Nov. 2008）

Index

薬学研究最前線

シグナル伝達プロテオームの最前線

石濱 泰[1,2]、杉山 直幸[1,3]（[1]慶應大・先端生命研、[2]JST さきがけ、[3]HMT）

1. はじめに

近年、質量分析計（MS）およびその周辺技術の急速な高性能化により、プロテオミクスはめざましい発展を見せている。最新の分析装置を用いれば、例えば、ヒト由来培養細胞で 1μg 程度の試料量があれば、約 2 時間の LC-MS 測定で、千種前後のタンパク質を恒常的に同定することができる。更に前分画法と組み合わせることにより、数千から一万タンパク質の網羅的発現解析を比較的簡単に行うことができるようになってきた。しかし、ゲノム配列解析や DNA マイクロアレイ測定の"網羅性"と比較すると依然としてプロテオミクスでカバーできる測定対象範囲は限られており、特に翻訳後修飾や細胞内局在、タンパク質相互作用なども含めたタンパク質の網羅的解析となると、上述のタンパク質発現解析のようにはいかないため、何らかの方法で選択的にタンパク質を濃縮あるいは分画して試料の複雑性やダイナミックレンジの幅を軽減してから、LC-MS 測定を行う必要がある。一方で、これら翻訳後修飾などの情報はプロテオミクスでのみ取得できるものであり、この分野の研究者は必要な技術開発を続けながらこれらの研究課題に取り組んでいる。筆者らは、中でも翻訳後修飾の最も一般的な修飾の一つであるタンパク質リン酸化に注目し、その網羅的解析のための技術開発を中心に研究を進めてきた。哺乳類では全タンパク質の 20-30%がリン酸化修飾を受けると言われており、リン酸化によりそのタンパク質の機能制御が行われている例も多い。特に細胞内シグナル伝達ネットワークにおいては、タンパク質キナーゼとその基質分子が中心的な役割を果たしている。増殖に関わるシグナル分子（タンパク質）が oncogene 産物であることがわかってきた 80 年代前半から多くの研究が行われ、様々な実験技術の発展とともに現在でも世界中で精力的に研究が行われている。今世紀に入ってからは分子標的薬といわれるシグナル分子キナーゼを標的とした阻害剤も次々と上市され、抗癌剤を用いた治療法にも大きな変化が起きている。しかし、細胞内シグナル伝達ネットワークは非常に複雑な制御システムを有しており、まだまだ多くの未知の部分が残されている。今日の分子標的薬の問題点である副作用や薬効の個人差といった原因のひとつには、この細胞内シグナル伝達ネットワークの複雑さが関与していると考えられる。このような複雑なネットワークを理解するには、個々の構成分子を一つひとつ調べていくのではなく、ネットワーク全体を一度に俯瞰できる方法が必須であり、プロテオミクスはまさにこれに適した方法であるといえる。

以下、筆者らが行ってきたプロテオミクスの手法を用いた網羅的タンパク質リン酸化解析法の開発について紹介する。また、この方法で得られた結果に基づいて、シグナル分子であるキナーゼとその基質の全体像や分子標的薬の評価システムとしての可能性についても述べる。

2. リン酸化ペプチド濃縮

MS を用いたプロテオミクスでは、タンパク質のままでは感度の点で不利であり、アミノ酸配列情報も取得できないため、トリプシンなどのプロテアーゼを用いてペプチドに切断してから MS 測定を行う。二次元電気泳動などのタンパク質レベルでの分離法を用いないショットガンプロテオミクスでは、複雑なタンパク質混合物のトリプシン消化物を、ミクロ化して感度を向上させたナノ LC とオンライン接続したタンデム MS（nanoLC-MS/MS）で直接測定する。このため、例えばヒト細胞試料の場合、その測定対象分子種数は優に 300 万を越え、そのダイナミックレンジは 10^6 以上となる[1]。リン酸化部位を含むペプチド（リン酸化ペプチド）は、このままの状態では大量に含まれる非リン酸化ペプチドに完全にマスクされてしまい、まったく検出されない。したがって、トリプシン切断後に選択的にリン酸化

ペプチドのみを濃縮することが必須となる（図1）。

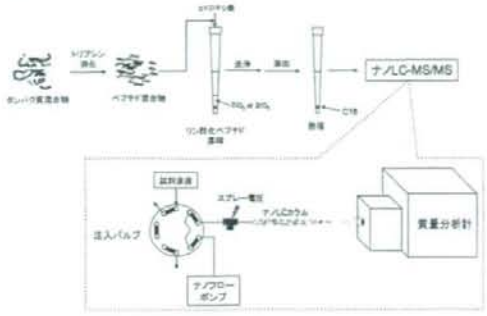

図 1　HAMMOC 法を用いたリン酸化プロテオミクス測定システム

リン酸化チロシン含有ペプチドの場合には、リン酸化チロシン抗体による濃縮が可能であるが、全リン酸化ペプチドの 90%以上を占めると考えられるリン酸化セリン、リン酸化スレオニンに対する特異性の高い抗体は今のところ存在しない。現在最も一般的なのは、リン酸基と鉄(III)・ガリウム(III)などの金属イオンとの親和性を利用した固定化金属アフィニティークロマトグラフィー（immobilized metal affinity chromatography, IMAC）またはチタニア、ジルコニアなどの酸化金属との親和性を利用した酸化金属クロマトグラフィー(metal oxide chromatography, MOC)を用いるものである。これらの化学的親和性を利用する方法は、リン酸化チロシン抗体ほどの特異性はないため、細胞全抽出物由来のペプチド混合物からリン酸化ペプチドを濃縮してくる際に必ず混入してくる非リン酸化ペプチドを除去する工夫が必要となる。この非リン酸化ペプチドは主として酸性ペプチドであり、親和性はリン酸化ペプチドよりは小さいが試料溶液中に大量に存在するために、結果としてリン酸化ペプチド濃縮の選択性を下げる原因となっていた。IMAC では、カルボン酸をメチルエステル化したり [2]、pH を最適化してリン酸基をイオン化させたままカルボン酸のみを中性化することにより、非リン酸化ペプチドの IMAC 樹脂に対する親和性を弱める試みが行われたが [3]、エステル化反応の反応率がばらついたり、リン酸基とカルボン酸基の pKa 値がペプチドによって異なり、リン酸化ペプチド濃縮の選択性を大きく改善することは難しかった。そこで、タンデム MS 内で、リン酸基特異的に生じるニュートラルロス反応を利用してリン酸化ペプチドに対する検出の選択性を上げたり [4]、ホスファターゼによる脱リン酸化反応を利用して、検索対象タンパク質範囲を限定させる必要があった [5]。一方、チタニアを用いた MOC では、最初のリン酸化ペプチド濃縮に関する報告以来 [6]、筆者らも含めていくつかのグループが、カゼインなどのリン酸化タンパク質の標準消化試料についてリン酸化ペプチドの濃縮が可能であることを示したが、細胞抽出物のような複雑な試料についての適用例の報告はなかった [7-9]。数年前、Larsen らは、2,5-ジヒドロキシ安息香酸やフタル酸といった芳香族カルボン酸を試料溶液中に共存させておくことにより、チタニア MOC におけるリン酸化ペプチドの濃縮選択性が向上することを報告した [10]。しかし、これらの芳香族カルボン酸は、ペプチドと逆相クロマトグラフィーにおける保持時間が重なり、LC-MS 測定の大きな障害となっていた。また、細胞抽出物の消化ペプチド混合物から直接リン酸化ペプチドを濃縮するには、その選択性は不十分であった。一方、谷らは、乳酸などのアリファティックなヒドロキシ酸がチタニアやジルコニアと安定な五員環キレートを形成し、強く保持されることを報告していた [11]。筆者らはこれに注目し、これらの親水性ヒドロキシ酸の酸化金属に対する親和性がリン酸基より弱く、酸性ペプチド中のカルボン酸や上記の芳香族カルボン酸よりも強ければ、ヒドロキシ酸で酸化金属を修飾することにより、細胞抽出物の消化ペプチド混合物からリン酸化ペプチドを直接濃縮できると考えた。また、ヒドロキシ酸は十分に親水性であり、LC-MS 測定前に通常の脱塩処理で除去可能であることから、LC-MS 測定に及ぼす影響も最小限にできると考えた。数種の親水性ヒドロキシ酸をスクリーニングしたところ、(図 2) に示すように、チタニアには乳酸が、ジルコニアには 3-ヒドロキシプロピオン酸が修飾剤として最適の親和性を有しており、従来の芳香族カルボン酸を共存させたチタニア MOC や IMAC 法よりも高い選択性でリン酸化ペプチドを濃縮できることがわかった [12]。

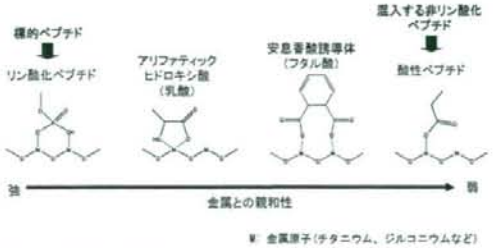

図 2　酸化金属担体とペプチドおよびヒドロキシ酸の推定相互作用様式

これらの方法は、世界で初めての細胞抽出物からリン酸化ペプチドを直接大量に濃縮できる方法であり、HAMMOC 法(Hydroxy Acid-Modified Metal Oxide Chromatography)と名づけた。（図3）は細胞全抽出物からのリン酸化ペプチド直接濃縮について、各方法を比較したものである。

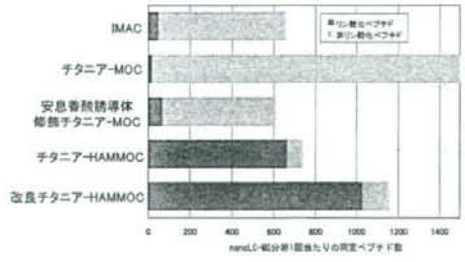

図 3　HeLa 細胞からのリン酸化ペプチドの濃縮
試料：50μg HeLa 細胞抽出物

乳酸修飾チタニアや 3-ヒドロキシプロピオン酸修飾ジルコニアを用いた HAMMOC 法とハイブリッド型イオントラップ-オービトラップ超高精度質量分析計を用いた nanoLC-MSMS 法を組み合わせることにより、50μg 程度の細胞抽出タンパク質試料から、1 回の LC-MS 分析で 500 個程度のリン酸化サイトを同定することが可能となった。その後、チタニア樹脂の焼成温度の最適化、表面の化学処理などの最適化を行ったところ、1 回の LC-MS 分析で 1000 個以上のリン酸化サイト同定が恒常的に可能となった。現在、これはリン酸化ペプチド濃縮キットとして市販されている。

上記のチタニアの最適化過程において、筆者らは入手可能な様々なチタニアを用いて、リン酸化ペプチド濃縮における性能を調べた。いずれのものについてもリン酸化ペプチドに対する親和性を示したが、ヒドロキシ酸に対する親和性との相対的な関係は一定ではなく、800℃以上で焼成したルチル型結晶のチタニアについては、ヒドロキシ酸非共存下でもリン酸化ペプチド濃縮の選択性がある程度高いことを見出した。この性質を利用し、チタニア-C18 の二相カラムを用いた全自動リン酸化ペプチド濃縮・分析システムも開発している[13]。

Bodenmiller らはショウジョウバエの細胞抽出液を用い、IMAC やチタニア MOC など 3 種類の代表的なリン酸化ペプチド濃縮法の比較を行い、現状では 1 つの方法でカバーできるリン酸化ペプチドは限定されているため複数の方法を相補的に使うのが望ましいと報告している[14]。しかし試料量が限られている場合などでは複数の方法を用いることは難しく、単一の方法で、より広い範囲のリン酸化ペプチドを同定できる方法が求められる。最近、筆者らは HAMMOC 法において溶出条件の最適化を行っている過程で、ピペリジンやピロリジンなどの環状アミンを用いた溶出溶液がその濃度によって異なる溶出選択性を示すことを見出し、この性質を用いて溶出ステップでリン酸化ペプチドを分画することにより、その回収率および同定数を大幅に向上させることに成功した[15]。現在では、例えばヒト子宮ガン由来 HeLa 細胞から抽出したタンパク質試料 100 μg から、3,000 以上のリン酸化サイトを同定することが恒常的に可能となっている。

さて、筆者らの研究室では昨年 8 月より、先に示した方法開発の過程やヒト由来培養細胞株を用いた他の研究プロジェクトを行いながら、得られたリン酸化サイト情報を蓄積してきた。現在最もよく使われている公共データベースの一つである UniProt Knowledgebase/Swiss-Prot（以下 SwissProt）に登録されているリン酸化サイトと比較した結果を（図 4）に示す。

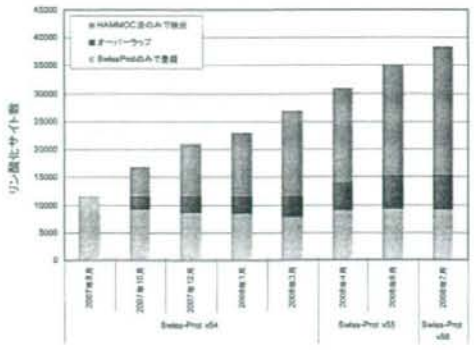

図 4　HAMMOC 法によって検出されたヒトタンパク質のリン酸化サイト数

一研究室での成果が世界中の研究成果の合計よりも2倍以上多い結果となっており、HAMMOC法がいかに優れた性能を持っているかを示すデータのひとつとなっている。また、今のところリン酸化サイト数の増加割合はいっこうに鈍る気配もみせておらず、SwissProt とのオーバーラップも緩やかながら着実に進んでいることから、今後も同定サイト数はこのペースで増加するものと思われる。なお、筆者らの結果については、MS/MS スペクトルを含めて現在公表する準備を進めている（論文化されたものについてはすでに PepBase（http://pepbase.iab.keio.ac.jp）にて公開中）。

HAMMOC 法の応用例として、最近筆者らは植物のリン酸化プロテオーム解析を行った[16]。モデルとしてシロイヌナズナ培養細胞株を用いて網羅的解析を行い、チロシンリン酸化が哺乳動物と同様の割合で存在していることを明らかにした。哺乳動物タイプのチロシンキナーゼがシロイヌナズナのゲノムにコードされていなかったことからその存在が疑問視されていた植物のチロシンリン酸化であるが、今回確認されたリン酸化チロシンプロテオームにより、哺乳動物タイプのチロシンキナーゼモチーフとは異なったユニークなモチーフが明らかとなり、植物特有のチロシンキナーゼファミリーの存在が示唆された。

3. タンパク質キナーゼとリン酸化プロテオミクス

ヒトタンパク質キナーゼの全容（キノーム，kinome）は Manning らによると約 500 とされる[17]。現在、SwissProt にはヒトのタンパク質キナーゼが 488 個、リン酸化情報が得られているタンパク質すなわちタンパク質キナーゼの基質と考えられるタンパク質が 5763 個、登録されている。多くのタンパク質キナーゼは、他のタンパク質キナーゼの基質になったり、自己リン酸化を起こすことが知られているため、SwissProt 登録タンパク質キナーゼ 488 個のうち、リン酸化タンパク質として登録されているものを調べてみたところ、394 個（81%）であった。残り 94 個のキナーゼのうち HAMMOC 法によりリン酸化タンパク質であると実験的に証明されたものが現在のところ 31 個あり、合計すると約 9 割のタンパク質キナーゼはリン酸化を受けていることとなる。この 425 個のリン酸化を受けるタンパク質キナーゼのうち、現在までに HAMMOC 法で同定されているものは 311 個あり、これはタンパク質キナーゼ全体に対して 64%、リン酸化を受けるタンパク質キナーゼに対して 73%となる（表 1）。

表 1　タンパク質キナーゼのリン酸化

	タンパク質数
Swiss-Prot v55.6に登録されているタンパク質キナーゼ	488
リン酸化されているタンパク質キナーゼ*	425
HAMMOC法で同定されたタンパク質キナーゼ**	311

* Swiss-Prot登録数とHAMMOC法による実測数より算出
** リン酸化タンパク質として検出

今後、様々な実験条件下でリン酸化プロテオーム解析を行うことでタンパク質キナーゼのうちリン酸化を受けるタンパク質の割合および HAMMOC 法によるタンパク質キナーゼの同定数はさらに増加すると考えられる。HAMMOC 法によって同定されたリン酸化サイトとそのキナーゼ活性との間に相関があるとは限らないが、これらのデータは、HAMMOC 法がキノーム基質のプロテオームだけでなく、キノーム自体を含んだ形での網羅的解析法であり、シグナル伝達ネットワークを記述する上で十分な情報量を提供できることを示唆している。

タンパク質キナーゼの推定総数と、筆者らの解

析結果で示されたタンパク質キナーゼ/基質比から考慮すると最終的には少なくとも 10,000 個以上のタンパク質および 50,000 個以上のリン酸化サイトがタンパク質キナーゼによってリン酸化されていると推定される。

4. おわりに

測定技術の進歩により、リン酸化プロテオーム解析はこの2,3年で驚くべきほどの進歩を遂げている。筆者らの開発した HAMMOC 法も含めていくつかの方法はすでにキット化され市販され始めており、数年の後にはより一般的な測定法になるものと思われる。筆者らも、より少ない細胞数試料から 1 万個レベルのリン酸化サイトの同定・定量が可能な高性能 HAMMOC 法の開発を更に進めており、これが実現すれば、現在 in vitro キナーゼアッセイによってプロファイル解析がされているキナーゼに対する分子標的薬評価についても、HAMMOC 法で可能になるであろう。In vivo 反応をモニターできるだけではなく、標的キナーゼを含めたパスウェイへの影響を評価でき、さらには未知の標的キナーゼを含めたシグナル伝達ネットワーク全体への影響を評価できるものと考えている。Olsen らによる EGF 刺激による動態実験 [18] のように安定同位体標識法と組み合わせることで、定量的な時間経過も測定可能であり、薬物の濃度依存性も評価できるため、今後の展開が期待できる。

参照文献

1. Ishihama, Y., Proteomic LC-MS systems using nanoscale liquid chromatography with tandem mass spectrometry. *J Chromatogr A* **2005,** 1067, (1-2), 73-83.
2. Ficarro, S. B.; McCleland, M. L.; Stukenberg, P. T.; Burke, D. J.; Ross, M. M.; Shabanowitz, J.; Hunt, D. F.; White, F. M., Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae. *Nat. Biotechnol.* **2002,** 20, (3), 301-5.
3. Kokubu, M.; Ishihama, Y.; Sato, T.; Nagasu, T.; Oda, Y., Specificity of immobilized metal affinity-based IMAC/C18 tip enrichment of phosphopeptides for protein phosphorylation analysis. *Anal. Chem.* **2005,** 77, (16), 5144-54.
4. Beausoleil, S. A.; Jedrychowski, M.; Schwartz, D.; Elias, J. E.; Villen, J.; Li, J.; Cohn, M. A.; Cantley, L. C.; Gygi, S. P., Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc. Natl. Acad. Sci. U S A* **2004,** 101, (33), 12130-5.
5. Ishihama, Y.; Wei, F. Y.; Aoshima, K.; Sato, T.; Kuromitsu, J.; Oda, Y., Enhancement of the efficiency of phosphoproteomic identification by removing phosphates after phosphopeptide enrichment. *J Proteome Res* **2007,** 6, (3), 1139-44.
6. Ikeguchi, Y.; Nakamura, H., Determination of Organic Phosphates by Column-Switching High Performance Anion-Exchange Chromatography Using On-Line Preconcentration on Titania. *Anal. Sci.* **1997,** 13, (6), 479-483.
7. Ishihama, Y.; Mann, M., Development of nanoLC-MS/MS systems for proteomics. *Chromatography (in Japanese)* **2003,** 24 (suppl.1), 12-13.
8. Kuroda, I.; Shintani, Y.; Motokawa, M.; Abe, S.; Furuno, M., Phosphopeptide-selective column-switching RP-HPLC with a titania precolumn. *Anal. Sci.* **2004,** 20, (9), 1313-9.
9. Pinkse, M. W.; Uitto, P. M.; Hilhorst, M. J.; Ooms, B.; Heck, A. J., Selective isolation at the femtomole level of phosphopeptides from proteolytic digests using 2D-NanoLC-ESI-MS/MS and titanium oxide precolumns. *Anal Chem* **2004,** 76, (14), 3935-43.
10. Larsen, M. R.; Thingholm, T. E.; Jensen, O. N.; Roepstorff, P.; Jorgensen, T. J., Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol. Cell. Proteomics* **2005,** 4, (7), 873-86.