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Identification and Validation of Eukaryotic Aspartate and Glutamate Methylation in Proteins

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Abstract

Methylation of lysine and arginine is known to be critical in cellular processes. However, methylation of other amino acidic residues has been largely overlooked. Here, we report a systematic screening for methylation of side chains of aspartate and glutamate (D/E-methylation), involving exhaustive nano-HPLC/MS/MS, a protein sequence database search, and manual verification. The putative D/E-methylated peptides were confirmed by MS/MS of synthetic peptides. Our analysis identified several D/E-methylation substrate proteins and their modification sites in human and yeast cells. To our knowledge, this is the first report conclusively identifying in vivo D/E-methylation substrates and their modification sites in eukaryotic cells, demonstrating that D/E-methylations are abundant protein modifications. The substrate proteins identified here provide a stepping stone for future biochemical characterization of protein methylation pathways.

Keywords

methylation; methylation of aspartic acid; methylation of glutamic acid; mass spectrometry; proteomics

Introduction

Post-translational modifications (PTMs) represent a major vehicle to diversify a cellular proteome. A protein can potentially be modified by diverse post-translational modifications that are catalyzed by enzymes encoded by about 5% of the genome in higher eukaryotes.[1,2] Substoichiometric, combinatorial covalent modifications among multiple sites of a protein sequence could lead to hundreds or even millions of possible molecular variants of a protein, with possible variant structure and fine-tuned functions. While much is known about several intensively studied PTMs (e.g., phosphorylation and ubiquitination), the functional consequences and substrate identities for many known PTMs remain largely unknown. Elucidation of the substrates for a PTM is usually one of the first few steps toward functional characterization and molecular dissection of a modification pathway.

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Supporting Information Available: Supplemental Figure S1. Comparison of spectra from experimentally identified D/E-methylated peptides, their corresponding synthetic peptides, and their corresponding non-methylated isoforms. Supplemental Table T1. A list of the number of identified proteins, peptides, and unique peptides for each HeLa and yeast sample in this study. This material is available free of charge via the internet at http://pubs.acs.org.

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Protein methylation catalyzed by *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase represents a major group of post-translational modifications.3 Methylation of lysine and arginine at histones is known to play an important role in the regulation of epigenetics and diseases.4 However, methylation of other ribosomally coded amino acid residues has not yet been carefully examined. In addition to arginine and lysine, methylation has been found at other amino acid residues such as aspartate (D), glutamate (E), histidine, asparagine, glutamine, and cysteine residues.1 Glutamate methylation has been shown to modulate chemotactic responses in *Escherichia coli*.[5] Nevertheless, definitive evidence of E-methylation in eukaryotes is lacking and no eukaryotic E-specific methyltransferase has been reported.[6] D-methylation has been reported in both eukaryotic and prokaryotic organisms, where it has been implicated as a protein repair mechanism.[6] However, the modification sites in D-methylated substrates have not been fully established. Given the importance of aspartate and glutamate in protein folding and functions, it is expected that neutralization of the amino acids' negative charge, an event similar to dephosphorylation, would have a significant impact on the proteins in a fashion similar to protein phosphorylation and lysine acetylation. Nevertheless, the substrates, functional consequences, and regulatory enzymes of D/E-methylation remain unknown.

Here we report a proteomics screening of D- and E-methylation by exhaustive identification of D- and E-methylated peptides and mapping of their corresponding modification sites by nano-HPLC/MS/MS analysis of *Saccharomyces cerevisiae* and HeLa cells (Figure 1). The methylated peptides were confirmed by manual verification and MS/MS of synthetic peptides. Our results suggest that D/E-methylation is an abundant post-translational modification, potentially occurring in about 2% of proteins in eukaryotic cells. Given the important roles of D/E residues in protein structure and function, as well as significant structural change induced by methylation, it is expected that D/E-methylation is likely to have a greater impact on the functions of its substrate proteins than lysine and arginine methylation. Identification of the methylation substrate proteins provides a stepping stone toward functional studies of the modification pathways and identification of their regulatory enzymes.

Results

Proteomics Screening of D/E-Methylation. Identification of D/E-Methylated Proteins

To test whether D/E-methylated proteins exist in vivo, we carried out a proteomics screening to identify D/E-methylated proteins and to evaluate the abundance and substrate diversity of the modifications in both *S. cerevisiae* and HeLa cells.

Identification of D/E-methylated proteins was carried out by exhaustive HPLC-MS/MS analysis of tryptic digests and protein sequence database searching (Figure 1). Briefly, a 40-μg protein whole-cell lysate from each cell type was resolved by SDS-PAGE. Each lane was divided into 20 bands of equal size. Each band was in-gel digested with trypsin, and the resulting tryptic peptides were analyzed by nano-HPLC/Orbitrap mass spectrometry for exhaustive peptide identification. The side chains of aspartate and glutamate can potentially undergo methyl esterification in vitro through chemical reactions when methanol is used as a cosolvent in staining/destaining buffer or in-gel digestion buffers.[7] In light of this observation, the experimental protocol was designed in such a way that methanol was excluded from each step of the experimental procedure, including gel staining/destaining, in-gel digestion, and HPLC separation. Such a procedure avoids in vitro D/E-methylation.[7] The MASCOT search algorithm used the resulting MS/MS spectra to identify peptide candidates from a protein sequence database. Two variable modifications, D-methylation and E-methylation, were included in the protein sequence database search.

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Validation Protocol for Peptide Identification

All the positive identifications were manually inspected to ensure the quality of analysis.[8] Since methylation could be present in a variety of other amino acid residues, including arginine, asparagine, cysteine, histidine, serine, and lysine,[1,6] special care was taken during the manual verification to make sure the methylated residue was located in either aspartate or glutamate residues instead of other amino acid residues. Those methylated peptides with ambiguous methylation sites were removed from our list. All the putative D/E-methylated peptides were further confirmed by MS/MS of synthetic peptides, the gold standard for confirming a chemical identity. The MS/MS spectra of all the identified, methylated peptides are included in the Supporting Information. The raw spectrum of each methylated peptide is available upon request.

We realize that such a comprehensive validation procedure is critical to ensure the accuracy of peptide identification. This is especially true for identifying methylated peptides as the methylation could be potentially present at several amino acid residues with polar side chains.[1] For example, if we set a Mascot score of 40 as peptide cutoff score, 16 D- or E-methylated peptides were found in MS/MS data sets of 6616 tryptic peptides from *S. cerevisiae*, of which only one D/E-methylated peptide could be validated. Likewise, 16 methylated K/R peptides with MASCOT score higher than 40 were found in *S. cerevisiae*, and only one of them could be validated (Supporting Information).

Polymorphism of D/E-Methylated Peptides

D-Methylated residues have a molecular weight equivalent to glutamate; therefore, either the MS/MS of a D-methylated peptide or a D-to-E-mutation peptide can explain the same MS/MS spectrum. We took two steps to address this problem. We first looked for known polymorphisms at the modified D residue. BLAST searching was carried out against the NCBI's human or *S. cerevisiae* databases, substituting E for our putative methyl-D identifications to ensure that our identifications did not arise from known sequence variants. Those residues with known D-to-E mutations were removed from our list. Since many post-translationally modified peptides are present in low stoichiometry, both modified and unmodified peptides should be present in a sample, and their corresponding MS/MS spectra may be identified in the same HPLC-MS/MS run. Therefore, the identified D-methylated peptides could be paired with MS/MS spectra of their corresponding nonmethylated peptides. Identification of three pairs of D-methylated peptides and non-D-methylated peptides suggest that at least these proteins are indeed D-methylated rather than polymorphisms resulting from D-to-E mutations (Figure 2, and Supporting Information). We further confirmed the identification of D-methylated peptides by MS/MS of their corresponding synthetic peptides (Supporting Information).

D- and E-Methylated Substrate Proteins. D/E-Methylated Proteins in *S. cerevisiae*

Two putative D-methylated substrate proteins (a heat shock protein and a protein with unknown function) and one E-methylated protein (TDH3, a yeast glyceraldehyde-3-phosphate dehydrogenase ortholog) were identified in our screening. We found three differentially modified forms of a peptide unambiguously derived from TDH3, including one unmodified, one singly E-methylated, and one doubly E-methylated form. The E-methylated glutamate residue, E170 of TDH3, is well conserved from bacteria to humans, suggesting a possible importance of the residue and regulatory influence for the modification. It is interesting to note that previous two-dimensional SDS-PAGE analysis of the yeast TDH isozymes resulted in resolution of two forms of TDH3 that differed only in charge.[9] The difference in charge was not due to phosphorylation, since the enzyme could not be labeled in extracts from yeast grown in the presence of ^{32}P[9]. It is possible that methylation of glutamate residues may explain the observed charge variation.

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D- and E-Methylated Proteins in HeLa Cells

Analysis of proteomic data from HeLa cells revealed D-methylation among five proteins, cytokeratin 9, AHNAK, Rab8, importin-8, and transthyretin. The molecular weight of a D-methylated residue is the same as that of an E residue. Therefore, the D-methylated peptides can only be fully confirmed if their corresponding, unmodified peptide is identified. Such analysis confirmed the D-methylation at three of the six peptides, peptides 1, 3, and 4 (Table 1) from the HeLa cells. The peptides 2, 5, and 6 should be considered putative D-methylated peptides.

AHNAK was found to be methylated at two positions. This protein was originally identified in a screen for transcripts lost in neuroblastoma cells.[10] Its localization, and presumably function, varies depending on cell type, and it has been shown to be targeted to the plasma membrane in response to its phosphorylation by either protein kinase B or protein kinase C.[11,12] Although its function is currently unknown, it may serve as a platform for the integration of intracellular signals.[13]

We also identified one protein associated with vesicle transport as being D-methylated. Rab8 is a GTPase reported to be involved in cytoskeletal remodeling and in the regulation of intracellular trafficking.[14] The identification of D-methylated proteins involved in trafficking suggests that the modification may play a regulatory role in this process. The modification has the capacity to modulate complex formation and target recognition through alteration of electrostatic interactions. Such a scenario is reminiscent of the effects of lysine acetylation.[15]

Prevalence of D/E-Methylation

The data obtained from our unbiased screen for methylated proteins prompted us to estimate the abundance of methylation based on the ratio of the methylated peptides and the total peptides identified. From the HeLa samples, 7052 unique peptides were positively identified, of which three were fully established to be D-methylated. Thus, the abundance of D-methylation is estimated to be around 4 in 10 000 peptides (0.043% of peptides). If we assume an average protein size is about 60 KD and the protein will generate about 50 possible tryptic peptides, we estimate that ~2% of human proteins may be subject to D-methylation. Likewise, the abundance of E-methylation among *S. cerevisiae* proteins is estimated as 0.8%. It should be noted that this estimate only reflects the abundance of the modifications among the most abundant proteins and that the low number of identified peptides precludes a thorough statistical analysis.

This study also identified a single methylated lysine-containing peptide in *S. cerevisiae*. Lysine acetylation is known to be an abundant post-translational modification in mammalian cells.[15] However, no lysine-acetylation substrate peptide was identified in our screening due to either our sensitivity limit or low stoichiometry of the modification in the substrate proteins. Collectively, the observed abundance of methylated peptides is likely to be in the range of lysine/arginine methylation or lysine acetylation.

Conclusions

In this proteomics screening, we conclusively identified two E-methylation sites from one substrate protein from *S. cerevisiae* as well as three D-methylation sites from three substrates from HeLa cells. These methylation events have not been described in eukaryotic cells before. The identified substrates include proteins with diverse functions, implicating the modifications as potential regulatory modifications involved in multiple pathways.

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Identification of a number of D/E-methylated proteins was initially a surprise to us. Lipases are abundant proteins in cells, and pro-drugs take advantage of in vivo-labile ester bonds to improve the pharmacokinetics of drugs. Nevertheless, several lines of evidence suggest that D/E-methylation indeed occurs in vivo instead of in vitro. First, protein lysates from cells were prepared in denaturing buffer to prevent PTM reactions in vitro. Second, methanol was avoided in each step of our experimental procedure. When standard nonmethylated peptides containing both aspartate and glutamate were processed for in-gel digestion and HPLC-MS/MS analysis, neither residue was observed to be methylated. Finally, E-methylation has been described in bacteria,[16] and methylation at C-terminal carboxylic acids is known to be present in cellular proteins such as prenylated proteins and PP2A.[17–21]

D/E-Methylation possesses a few unique features to serve as an ideal vehicle for regulatory functions in eukaryotic cells. First, a large group of methyltransferases, demethylases, and esterases exist in cells, some of which might serve as enzymes for controlling the status of D/E-methylation. Second, neutralization of D/E side chains provides a versatile approach to change a protein's structure, interactions, and therefore its functions. Third, given the important roles of aspartate in enzymatic reactions, methylation of key aspartates in an active center is likely to alter the enzymatic activity of the substrate proteins. Fourth, D/E-methylation is known to be reversible.[6] Fifth, acidic residues are present in many recognition motifs of post-translational modifications, such as the sumoylation recognition motif (hydrophobic K*X*E, where *X* is any amino acid) and phosphorylation consensus motifs (e.g., casein kinase motif). Neutralization of D/E side chains may provide an extra layer of regulation for those post-translational modifications in tandem cascades. Finally, *S*-adenosyl-L-methionine is the most widely used enzyme substrate after ATP.[22] It is distributed among virtually all body tissues and fluids.

Methylation of arginine and lysine residues has been extensively studied in the past decade, especially on histone proteins. Previous studies demonstrated that methylation of arginine and lysine residues is reversible and involved in the regulation of gene expression by altering chromatin structure or by creating a binding platform to recruit transcriptional regulators.[23,24] D/E-Methylation changes three major properties of the substrate amino acid: neutralization of negative charge, increasing size, and hydrophobicity. Thus, D/E-methylation is like a dephosphorylation event in terms of charge change. In comparison with methylation of arginine and lysine, D/E-methylation leads to more significant structural changes because methylation of arginine and lysine residues has little effect on their side chain's charge and hydrophobicity. Consequently, D/E-methylation should elicit more dramatic alteration of their substrate proteins than methylation of arginine and lysine.

Protein structure is influenced to a large extent by the charge state of ionizable groups on the side chains of several amino acids.[25] Copious literature documents show a decrease in an amino acid's net charge, induced by post-translational modifications such as acetylation and phosphorylation, alter diverse protein properties. However, similar alterations in net charge among amino acid residues with negative charge (D and E) have been overlooked in the research community. Given the high prevalence and possible dynamic nature of D/E-methylation and the resulting dramatic changes in substrate residues' side chain properties, it is reasonable to assume that D/E-methylation could be a gold mine that remains to be explored by the biomedical research community.

It is important to note that our proteomics analysis of D/E-methylation did not involve prior enrichment for D/E-methylated proteins. Given that protein modifications often occur substoichiometrically, unmodified peptides would be expected to significantly outnumber modified peptides upon digestion, leading to suppression in the number of identified modifications. Our ability to identify E-methylated peptides even without prior enrichment

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suggests a vast frontier the exploration of which awaits development of suitable enrichment strategies. We realize that mass shifts of +14 Da have been observed at D/E residues for some tryptic peptides and were considered as D/E-methylated peptides in two independent reports.[26,27] However, no validation experiment was carried out in these reports. In addition, the possible polymorphism of D-to-E mutation was not checked in the case of the claimed D-methylation.[27] Therefore, the accuracy of the identification was not certain.

The data set of D/E-methylation demonstrates that these post-translational modifications are abundant in proteins, reminding us of the potentially critical roles of the modifications in cellular regulation. The striking data raise many interesting questions. What are the enzymes responsible for modulating the status of D/E-methylation? Which cellular pathways are regulated by D/E-methylation? Is this modification compartmentalized? How dynamic is the modification? Does D/E-methylation cross-talk with other post-translational modification pathways? How is the modification involved in the progression of disease? The D/E-methylation data sets generated in this study offer a stepping stone, driving experimental efforts toward these directions.

Experimental Procedures

Preparation of Cell Lysates from HeLa Cells and Yeast

One dish (10 cm) of HeLa cells was grown to 80–90% confluence. The cells were washed with cold PBS buffer twice, and then 0.3 mL of cell lysis buffer was added (2% SDS, 62.5 mM Tris-HCl, pH 6.8, protease inhibitors). The cell lysate was harvested and sonicated three times for 5 s each with 20-s intervals between sonications. The lysate was centrifuged at 4 °C for 1 h at 21 000*g*. The supernatant was considered HeLa-cell whole-cell lysate.

The *S. cerevisiae* 647 cells were grown in YPD medium at 30 °C for 24 h to midlog phase (1–5 × 10^7 cells/ml). Cells were harvested, washed with PBS, and resuspended in 0.2 mL of cell-breaking buffer (7 M urea, 2 M thio-urea, 4% CHAPS, 50 mM Tris-Cl, pH 8.0, 10 mM DTT and protease inhibiotors), and lysed with acid-washed glass beads (0.45 to 0.55 mm). The lysate was centrifuged at 4 °C for 1 h at 21 000*g*. The pellet was discarded, and the supernatant was considered *S. cerevisiae* whole-cell protein extract.

An amount of 40 *µ*g of yeast and HeLa whole-cell lysates were resolved in a 12% SDS-PAGE gel. The gel was stained using a Colloidal Coomassie staining kit. The resolved proteins were cut out into 20 pieces of equal size for in-gel digestion.

Isolation of GST Fusion Proteins

See Supporting Information for a detailed protocol.

Verification of in Vivo Methylation in Purified GST Fusion Proteins

TDH3 purified from *S. cerevisiae* was subjected to SDS-PAGE and stained with colloidal Coomassie blue. The protein band corresponding to TDH3 was cut from the gel and subjected to in-gel digestion and HPLC-MS/MS analysis as described below.

In-Gel Digestion

Gel bands were sliced into small pieces (~1 mm^3) and destained with 25 mM ammonium bicarbonate solution (ethanol/water, 50:50 v/v). The destained gel pieces were washed in an acidic buffer (acetic acid/ethanol/water, 10:50:40, v/v/v) three times for one hour each time and in water two times for 20 min each time. The gel pieces were dehydrated in 100% acetonitrile and dried in a SpeedVac (ThermoFisher, Waltham, MA). An amount on 150 ng of porcine modified trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate was

added to the dried gels and incubated overnight at 37 °C. Tryptic peptides were sequentially extracted from the gel pieces with 50% acetonitrile buffer (acetonitrile/water/trifluoroacetic acid (TFA), 50:45:5, v/v/v) and 75% acetonitrile buffer (acetonitrile/water/TFA, 75:24:1, v/v/v). The peptide extracts were pooled, dried in a SpeedVac, and desalted using a μ-C18 Ziptip (Millipore, Billerica, MA) prior to HPLC/MS/MS analysis.

HPLC-MS Analysis

Each sample was dissolved in 10 μL of HPLC buffer A (0.1% formic acid in water (v/v)), and 2 μL was injected into the Surveyor HPLC system (ThermoFinnigan, Waltham, MA) using an autosampler. Peptides were separated on a homemade capillary HPLC column (50 mm length × 75 μm ID, 4 μm particle size, 90 Å pore diameter) with Jupiter C12 resin (Phenomenex, St. Torrance, CA) and directly eletrosprayed into a mass spectrometer using a nanospray source. LTQ-Orbitrap was operated in the data-dependent mode acquiring fragmentation spectra of the six strongest ions. HPLC/LTQ mass spectrometer analysis was carried out as previously reported.[8]

Protein Sequence Database Search and Manual Verification

All MS/MS spectra were searched against the NCBI-nr protein sequence database with the specification of D- or E-methylation using the MASCOT database search engine. All D/E-methylated peptide identifications with a MASCOT score >35.0 were manually examined with the rules previously described before,[8] and all D/E-methylation sites have to be identified by consecutive b- or y-ions so that the possibilities that methylation or a +14 Da mass shift occurs on adjacent residues were eliminated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

D	aspartate
E	glutamate
PTM	post-translational modification
MS	mass spectrometry
MS/MS	tandem mass spectrometry

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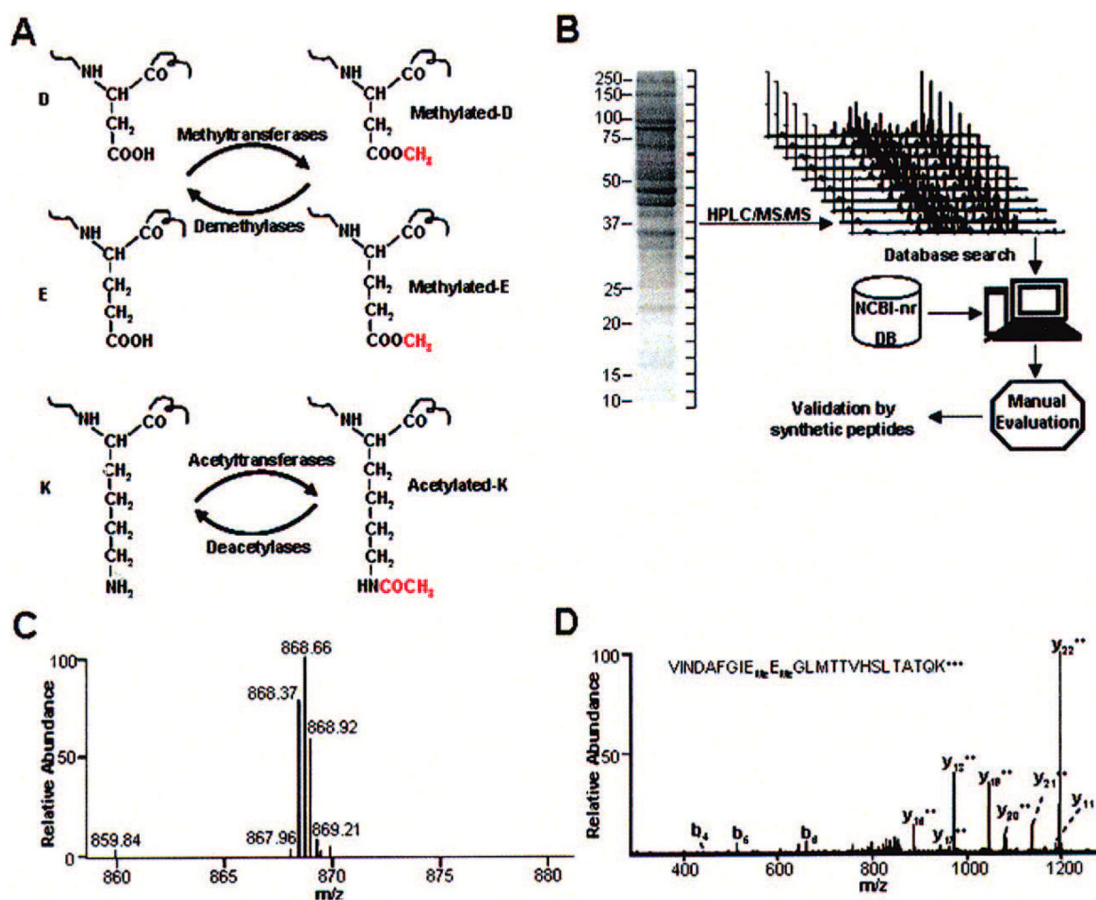

Figure 1.

Schemes for proteomic analysis of D/E-methylation. (A) Structural changes induced by D/E-methylation and lysine acetylation. (B) The strategy for proteomic screening of D/E-methylation. The whole-cell lysate of interest was resolved on SDS-PAGE, and 20 protein bands of equal width were excised and in-gel digested. The resulting peptides were analyzed by nano-HPLC/MS/MS, and the mass spectrometric data were searched against the NCBI-nr database by the MASCOT search engine for peptide identification and localization of D- or E-methylation sites. All candidate modified peptides were manually verified and then further confirmed by MS/MS of synthetic peptides. (C, D) MS zoom scan and MS/MS of an E-methylated peptide identified by a triply charged peptide of sequence "VINDAFGI**E*****E***GLMTTVHSLTATQK" (**E*** indicates methylated glutamic acid). b and y ions designate N- and C-terminal fragment ions produced by collision-induced backbone fragmentation in the mass spectrometer.

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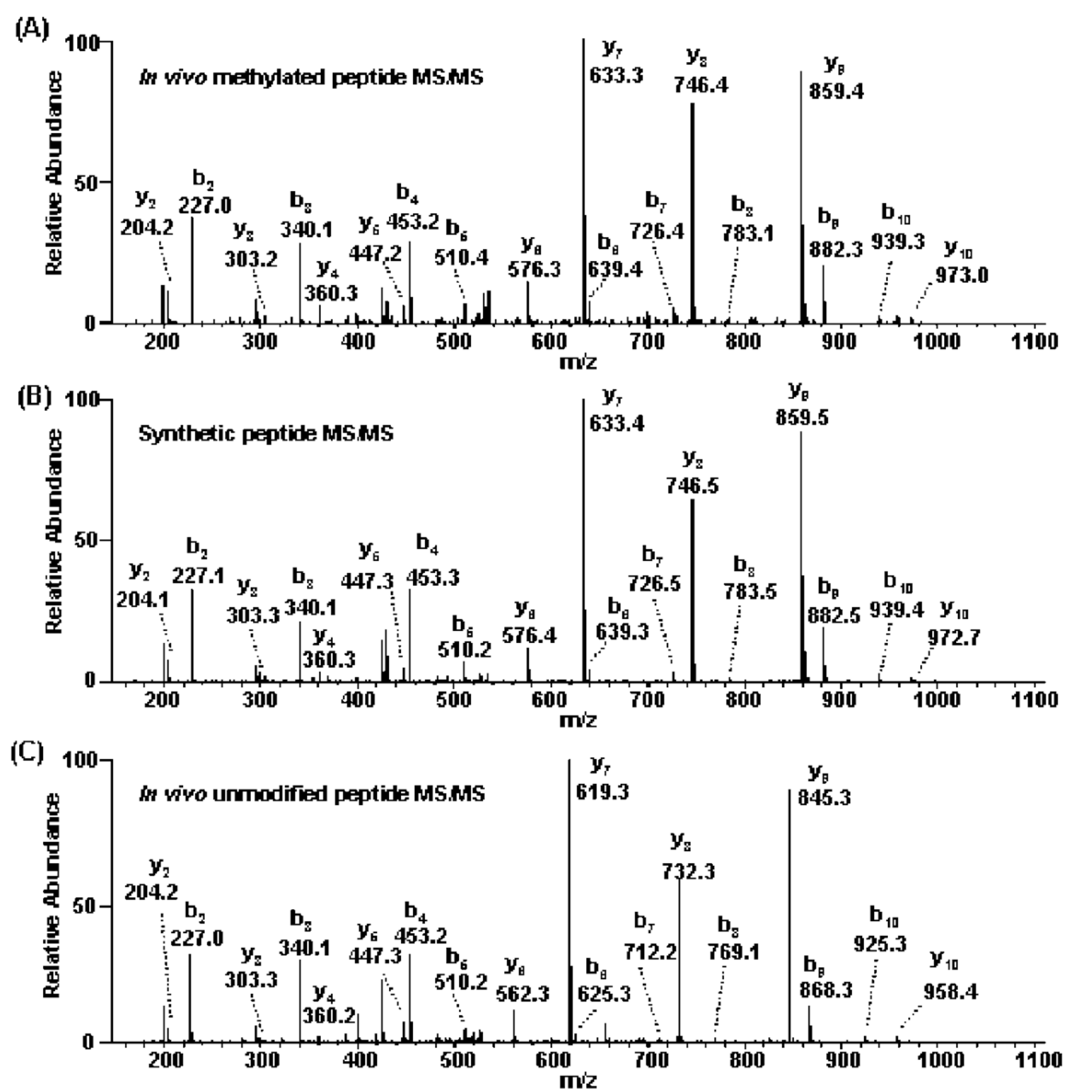

Figure 2.

Verification of a D-methylated peptide. (A) MS/MS of an in vivo D-methylated peptide "LLLIG**D***SGVGK" (**D*** indicates methylated aspartic acid), as well as (B) its corresponding synthetic peptide and (C) its unmodified isoform identified in vivo.

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Table 1

List of D- and E-Methylated Peptides Identified in this Screen[a]

no.	peptide	modified residue	protein name	GI number	identified peptide #	coverage	unmodified peptide
			Human				
1	VQALEEANN**D***LENK	D	cytokeratin 9	435476	16	36%	+
2	VDI**D***APDVSIEGPDAK	D	AHNAK	61743954	17	4%	−
3	ISMPDV**D***LHLK	D	AHNAK	61743954	17	4%	+
4	LLLIG**D***SGVGK	D	rab8	452318	5	28%	+
5	ETEND**D***VTNVIQK	D	importin-8	45477008	3	3%	−
6	AAD**D***TWEPFASGK	D	transthyretin	37483	1	8%	−
			Yeast				
7	VLGTAY**D***K	D	HSP	312611	3	4%	−
8	LIQ**D***ESTK	D	unknown	6323832	3	13%	−
9	VINDAFGI**E***__E__*GLMTTVHSLTATQK	E	TDH3	1323341	19	79%	+

[a]Methylation sites are denoted in bold, followed by an asterisk. In the “unmodified peptide” column, “+” indicates that the corresponding unmodified peptide was identified while “−” indicates that the unmodified peptide was not identified in the same HPLC-MS/MS analysis. The number of identified peptides and sequence coverage for each D/E-methylated protein is also included. MS/MS spectra of all D- and E-methylated peptides have been validated by their corresponding synthetic peptides (Supporting Information).