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Proteomic Analysis of Methylarginine-Containing Proteins in HeLa Cells by Two-Dimensional Gel Electrophoresis and Immunoblotting with a Methylarginine-Specific Antibody

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Abstract Protein arginine methylation is found in many nucleic acid binding proteins affecting numerous cellular functions. In this study we identified methylarginine-containing proteins in HeLa cell extracts by two-dimensional electrophoresis and immunoblotting with a methylarginine-specific antibody. Protein spots with matched protein stain and blotting signals were analyzed by mass spectrometry. The identities of 12 protein spots as 11 different proteins were suggested. Known methylarginine-containing proteins such as hnRNP A2/B1, hnRNP A1, hnRNP G and FUS were identified, indicating the feasibility of our approach. However, four highly abundant metabolic enzymes that might co-electrophorese with methylarginine-containing proteins were also identified. Other nucleic acid binding proteins hnRNP M, hnRNP I and NonO protein were identified. Recombinant hnRNP M and a peptide with the RGG sequence in hnRNP M could be further methylated in vitro. The immunoblotting results of immunoprecipitated hnRNP I and NonO protein are consistent with arginine methylation in both proteins. In this study we identified methylarginine-containing proteins in HeLa cells through proteomic approaches and the method is fast and robust for further applications.

Keywords Proteomic analysis · Posttranslational modification · RNA binding proteins · Methylarginine-containing proteins · Protein arginine methylation

Abbreviations

PTM	Posttranslational modification
PRMT	Protein arginine methyltransferase
AdoMet	S-adenosylmethionine
aDMA	Asymmetric dimethylarginine
sDMA	Symmetric dimethylarginine
MMA	Monomethylarginine
2-DE	Two-dimensional electrophoresis
IPG	Immobilized pH gradient
IEF	Isoelectrofocusing

1 Introduction

Posttranslational modification (PTM) to modulate the structure and function of proteins is an exciting field in proteomic analyses. Protein arginine methylation has become one of the most intensively studied PTM involved in various cellular functions such as signal transduction, protein subcellular localization, transcriptional regulation, protein–protein interactions, DNA repair [2, 25] and even embryogenesis [28]. By now at least 11 protein arginine methyltransferase (PRMT) genes have been identified in the mammalian system to catalyze the transfer of methyl groups from *S*-adenosylmethionine (AdoMet) to the side chain ω-guanido nitrogens of arginine residues in protein

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substrates [16]. The PRMT activity can be further divided into type I and type II, depending on the catalyses to form asymmetric di-ω-N^G, N^G-methylarginine (aDMA) or symmetric di-ω-N^G, N'^G-methylarginine (sDMA) residues, respectively [9].

Most of the methylarginines identified in proteins appear to be N^G-monomethylarginine (MMA) and aDMA in various RNA binding proteins within the Arg-Gly-Gly or Arg-Gly repeat context [2, 25]. The arginine methylaccepting substrates or methylarginine-containing proteins have been identified and studied through different approaches. Early biochemical analyses by amino acid analyses or Edman sequencing identified aDMA in proteins such as fibrillarin [21] and nucleolin [18]. In the recent years, different proteomic approaches have been used to analyze the modification. For example, putative methylaccepting proteins were identified by inhibiting general protein methylation in vivo and then modifying the hypomethylated proteins in vitro [12, 23]. Specific PRMT knock-out models also suggested specific sets of hypomethylated proteins which could be further in vitro methylated for analyses [8, 26, 31]. Furthermore, PRMT1 substrates were screened from a λgt11-HeLa cDNA library [29]. Substrates for PRMT1 and CARM1 (Coactivator-associated arginine methyltransferase 1)/PRMT4 were identified by solid-phase supported enzyme reactions with arrayed high-density protein membranes [19]. In addition, methylarginine-containing proteins were identified using stable isotope labeling by amino acids in cell culture (SILAC) [24]. The list of arginine methylaccepting substrates for specific PRMT enzymes are growing very fast as reviewed [2, 25].

Protein phosphorylation is the most studied PTM and antibodies against phosphorylated amino acids have greatly facilitated the study of phospho-proteome. Detection of phosphorylated proteins by Western blot of two-dimensional polyacrylamide gels has been developed [14]. A few arginine-specific antibodies for the recognition and identification of methylarginine-containing proteins are commercially available. Identification of arginine-methylated proteins proteomically by immunoprecipitation with methylarginine-specific antibodies has been conducted [3]. However, proteins interacting with methylarginine-containing proteins might be co-immunoprecipitated. Stable protein methylation in HeLa cells has been illustrated and a mono- and di-methylarginine antibody 7E6 detected numerous proteins in HeLa extract through standard one-dimensional immunoblots [4]. In this study we thus utilized 7E6 to detect the methylarginine-containing proteins in HeLa cells proteomically. We separated HeLa cell proteins by two-dimensional electrophoresis (2-DE) and compared the pattern of the stained proteins with that of the Western blot signals to identify specific methylarginine-containing proteins.

2 Materials and Methods

2.1 Cell Cultures and Protein Extraction

HeLa cells (ATCC CCL-2) grown in a 75 mL culture flask were washed with phosphate buffer saline (PBS), scraped on ice in PBS, collected by centrifugation and then resuspended in 200 μL of rehydrotion buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% IPG buffer pH 6–11, and 2.5% dithiothreitol (DTT). Cells were incubated at room temperature for 30 min. Lysed cells were subjected to centrifugation at 12,000*g* for 20 min at 4 °C and the supernatant was collected as the cell extract. Proteins in the extracts were quantified by 2-D quant kit (GE Healthcare) with bovine serum albumin as the standard.

2.2 Two-dimensional Gel Electrophoresis

Immobilized pH gradient (IPG) strips (pH 6–11, 7 cm, GE Healthcare) were rehydrated with gels facing down at room temperature for 16 h in modified rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% IPG buffer pH 6–11, 10% isopropanol, 5% glycerol, 2.5% DTT) for isoelectrofocusing (IEF) electrophoresis at alkaline pH as suggested by Görg et al. [10] and Hoving et al. [11]. The rehydrated strips were then transferred to the Ettan IPGphor cup loading manifold with the gel-side facing up. Protein samples (250 μg) were loaded on the strips at the anodic side. IEF were carried out in an IPGphor system as instructed by the manufacturer (GE Healthcare). Upon the completion of IEF (13,350 volt-hours), the strips were equilibrated in equilibration buffer I (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% (w/v) SDS, 1% DTT) for 15 min, and in equilibration buffer II (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% (w/v) SDS, 135 mM iodoacetamide) for another 15 min. SDS polyacrylamide gels (10%) were employed for the second dimensional separation in a Mini-PROTEAN III system (Bio-Rad).

2.3 Western Blotting

Protein samples separated by the above 2-D electrophoresis or from immunoprecipitation were transferred to nitrocellulose membranes The membrane was then blocked, incubated with specific primary antibody 7E6 (1:200 dilution; Abcam), anti-hnRNPI or anti-NonO (1:500 dilution; Santa Cruz), washed two times in TTBS (20 mM Tris–HCl, pH 7.5; 150 mM NaCl; 0.05% Tween 20), and then incubated with secondary antibody (IgG horse radish peroxidase conjugate; anti-mouse for 7E6 or anti-hnRNPI; anti-goat for anti-NonO). Chemiluminescent detection was

performed using the Supersignal kit (Pierce) or the Vis-Glow chemiluminescent substrate (Visual Protein).

2.4 In Gel Protein Digestion

In gel digestion was performed using the Montáge^{TM} In-Gel Digest$_{ZP}$ Kit (Millipore). Basically, protein spots were excised from the gel with pipette tips and the gel plugs were placed in the ZipPlate plate. The gel plugs were destained, dehydrated and trypsinized. The digested peptides were then extracted, captured and eluted as suggested by the manufacturer.

2.5 Mass Spectrometry and Protein Identification

The eluted peptides were vacuum dried and analyzed by Core Facilities for Proteomics Research in the Institute of Biological Chemistry, Academia Sinica. Protein spots were subjected to concerted MALDI peptide mass fingerprinting (PMF) and CID MS/MS analysis for protein identification using a dedicated Q-Tof Ultima^{TM} MALDI instrument (Micromass, Manchester, UK) operated under MassLynx 4.0. For MALDI MS and MS/MS analysis, samples were premixed 1:1 with matrix solution (5 mg/mL CHCA in 50% acetonitrile, 0.1% v/v TFA and 2% w/v ammonium citrate). Parent ions meeting the predefined criteria (any peak within the *m/z* 800–3,000 range with intensity above 10 count ± include/exclude list) were selected for CID MS/MS using argon as collision gas and a mass dependent ±5 v rolling collision energy.

The MS or MS/MS spectra data were analyzed by Mascot mass fingerprinting and MS/MS ion search (http://www.matrixscience.com) with the following characteristics: peptide mass fingerprinting: database: NCBInr; taxonomy: *Homo sapiens*; enzyme: trypsin; fixed modification: carbamidomethyl (C); variable modifications: oxidation (M), dimethyl (R), methyl (R); max missed cut: 1 (increased to 3 when dimethyl or methyl arginine modification was considered); peptide tolerance: 0.1 Da; mass values: MH^+. MS/MS ion search: database: NCBInr; taxonomy: *Homo sapiens*; enzyme: trypsin; max missed cut: 1; fixed modification: carbamidomethyl (C); variable modifications: oxidation (M); peptide tolerance: 0.1 Da; MS/MS tolerance: 0.1 Da; peptide charge: 1+; data format: Micromass (.PKL); instrument: MALDI-QUAD-TOF.

2.6 Plasmid Construction and Purification of GST-fusion Proteins

The protein coding region for hnRNPM were amplified by polymerase chain reaction from cDNA clones from RZPD (Germany) with the primer sets containing a *Bam HI* site in the forward primer and a *Not I* site in the reverse primer. The PCR products were ligated into a TA vector and the desired insert was then cloned into pGEX4T1. Expression of the recombinant glutathion-*S*-transferase (GST)-fusion protein in DH5α was induced by 1 mM IPTG. GST fusion proteins were purified with Glutathione-Sepharose 4B (GE Healthcare).

2.7 In vitro Methylation Reaction and Fluorography

Recombinant protein or synthetic peptides were incubated with [*methyl*-^{3}H]-AdoMet (60 Ci/mmol, GE Healthcare) in the presence of arginine methyltransferase source in Buffer B (50 mM sodium phosphate, pH 7.5). The reaction was carried out at 37 °C for 60 min, terminated by the addition of one-third of the volume of 4× SDS sample buffer, and the samples were subjected to SDS–PAGE (12.5% acrylamide) as described by Laemmli [17]. The gels were stained with Coomassie brilliant blue, destained, treated with EN^{3}HANCE (Perkin Elmer), dried and exposed to X-ray film (Kodak, MS) at −75 °C. Recombinant rat GST-PRMT1 was prepared as previously described [20]. Porcine brain extract was prepared as described in [13] and the S3 porcine brain extract is the supernatant of ultracentrifugation at 130,000*g* for 1 h.

2.8 Immunoprecipitation

HeLa cell extract (750 μg of protein) prepared as described in [4] was incubated overnight at 4 °C with 3 μg of antibodies (anti-hnRNPI or anti-NonO) and 40 μL of protein G-Sepharose beads (GE Healthcare) in PBS buffer containing 5% glycerol, 1 mM EGTA, 1× Complete EDTA-free protease inhibitor cocktail and 0.5% Triton-X-100. The beads were then washed for three times in the same buffer. Proteins bound to the beads were eluted by boiling in the SDS–PAGE sample buffer and subjected to Western blot analysis.

3 Results

3.1 Separation and Detection of Methyl-Arginine Containing Proteins by 2-DE Followed by Western Blot Analyses with a Methylarginine Specific Antibody

As most known methylarginine-containing proteins are basic proteins with high p*I* values, we separated the HeLa protein extracts with pH 6-11 IPG strips for the first dimensional IEF electrophoresis. A 2-D electrophoresis was performed in duplicates. One gel was transferred to nitrocellulose membrane followed by immunoblotting with

the methylarginine-specific antibody 7E6 (Fig. 1a) and the other was stained with Coomassie blue (Fig. 1b). As shown in Fig. 1a, the strongest signals detected by the antibody are a series of spots within pH 8.5–10 at the molecular mass around 36 kDa. Other strong signals are streaks around molecular mass of 75–80 kDa with p*I* value around 7–8 and 9–10. A few large dots that should contain more than one protein were also detected. Thirty-five Coomassie-stained protein spots can be correlated with the signals in immunoblots. These spots are likely to be or contain methylarginine-containing proteins in HeLa cells.

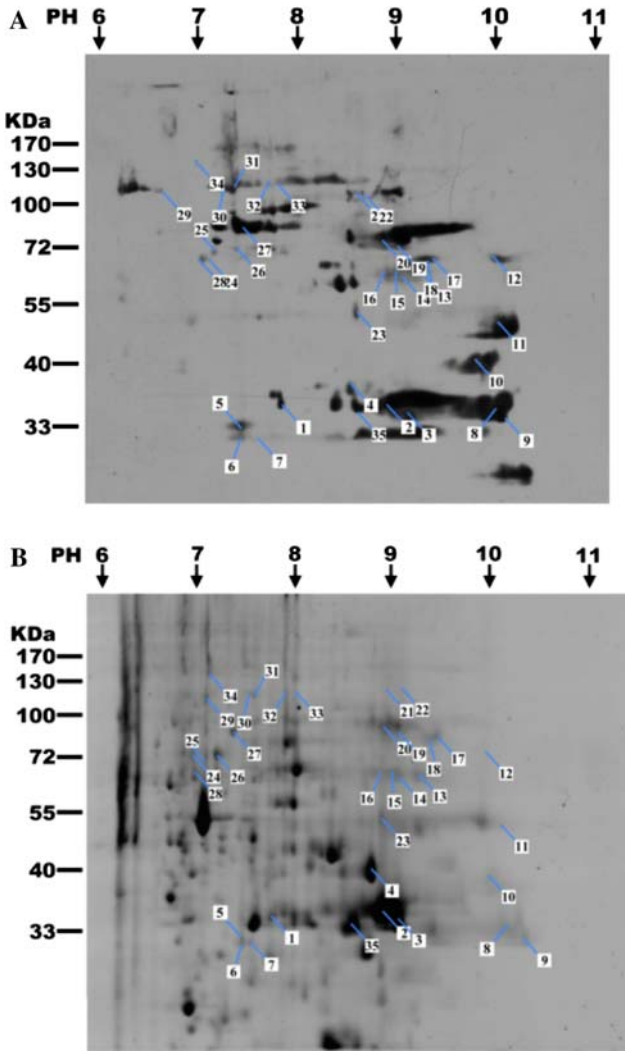

Fig. 1 Methylarginine-containing proteins in HeLa cells separated by 2-DE and detected by Western blot. HeLa cell extract (250 μg) was separated by IEF electrophoresis (pH 6–11) and then SDS–PAGE in duplicates. **a** The methylarginine-containing proteins in one 2-D gel were detected by methylarginine-specific antibody 7E6. **b** The proteins in another duplicate gel were stained by Coomassie blue. The proteins spots with corresponding immunoblot signals were indicated and numbered

3.2 Identification of the Methylarginine-Containing Proteins

We further analyzed the protein spots that could be correlated with signals detected by methylarginine-specific antibody with p*I* values above pH 8. The spots were excised, protease-digested and then subjected to mass spectrometric analyses. The identities of 11 spots were suggested by Mascot analyses of the MS as well as the MS/MS data (Table 1). Among these, three proteins are suggested for one intensely stained protein spot (spot 3), and two spots (spot 15 and 16) were identified as the same protein. We identified four proteins with typical RGG motifs with verified arginine methylation sites. These proteins include hnRNPA1 [15, 24, 30], hnRNPA2/B1 [12, 24], hnRNP G [24] and FUS protein [24, 27]. If the Mascot search parameters were less strict, another typical methylarginine-containing protein Sam 68 [6, 24, 27] could also be identified with a low score. Three other nucleic acid binding proteins hnRNPI, hnRNPM and NonO protein (54 kDa nuclear RNA- and DNA-binding protein, p54/nrb) were identified. A previous analysis of protein methylation in hnRNP complex showed that hnRNPM and hnRNPI were weakly methylated in vitro but not in vivo [22]. Four spots corresponding to highly abundant protein signals were identified as metabolic enzymes: glyceraldehyde 3-phosphate dehydrogenase, malate dehydrogenase, aldolase and lactate dehydrogenase. Mascot analyses of the MS/MS data of spot 3, an enormous protein spot, identified peptides from three different proteins with ion scores higher than those indicating identity. The proteins are malate dehydrogenase, hnRNPA2/B1 and glyceraldehydes 3-phosphate dehydrogenase with two, one and one peptide unambiguously indicated their identities.

An online program MeMo to predict lysine or arginine methylation sites was designed based on the known methylation sites in proteins [5]. Arginine methylation sites predicted by MeMo in the putative methylarginine-containing proteins were listed in Supplementary Table 1. Multiple putative methylation sites are predicted for the three nucleic acid binding proteins. No methylation sites could be suggested for aldolase and lactate dehydrogenase while two and one putative arginine methylation sites were suggested for glyceraldehyde-3-phosphate dehydrogenase and malate dehydrogenase, respectively.

3.3 Suggestion of Methylarginine Sites in hnRNPA1 by the MALDI-TOF Mass Spectrometric Data

When the peptide mass fingerprint analyses of the MALDI-TOF MS data were analyzed by Mascot with mono- or di-methylation of arginine as the variable modification in the search parameters, multiple peptides with putative

Table 1 Proteins matched to the methylarginine signals detected by methylarginine-specific antibody 7E6 identified by MS/MS

Spot number	Identified protein	NCBI accession number	Theor. Mr/ept. Mr (kDa)	Theor. p*I*/exp. p*I*	Mascot score[a]	Identified peptides	Ion score/expect[b]	Sequence coverage (%)
2	Glyceraldehyde-3-phosphate dehydrogenase	gi|31645	36/37	8.4/8.9	149	LVINGNPITIFQER	73/1.9e−05	8
						LISWYDNEFGYSNR	76/8e−06	
3	a. Malate dehydrogenase	gi|6648067	37/36	9.0/9.1	142	VAVLGASGGIGQPLSLLLK	44/0.00051	12
						LTLYDIAHTPGVAADLSHIETK	98/1.6e−08	
	b. Heterogeneous nuclear ribonucleo-proteins A2/B1 (hnRNP A2/B1 isoform B1)	gi|133257	37/36	9.0/9.1	114	LFIGGLSFETTEESLR	114/16.8e−10	4
	c. Glyceraldehydes-3-phosphate dehydrogenase	gi|31645	36/36	8.3/9.1	44	LVINGNPITIFQER	44/0.005	4
4	Aldolase A	gi|229674	39.7/41	8.4/8.7	298	ADDGRPFPQVIK	39/0.03	19
						VNPCIGGVILFHETLYQK	64/4.2e−05	
						YTPSGQAGAAASESLFVSNHAY	86/2.4e−07	
						IGEHTPSALAIMENANVLAR + Oxidation (M)	108/2.1e−09	
8	Heterogeneous nuclear ribonucleoprotein A1 isoform a (hnRNP A1)	gi|4504445	34/35	9.3/10.2	271	SSGPYGGGGQYFAKPR	86/4.5e-07	14
						GFAFVTFDDHDSVDK	72/7.5e−06	
						LFIGGLSFETTDESLR	113/9.8e−10	
11	hnRNP G	gi|542850	47/52	9.6/10.1	57	AIKVEQATKPSFESGR	28/7.4	7
						DSYGGPPRREPLPSRR*(3 miss cut)	25/15	
13	Heterogeneous nuclear ribonucleo-protein I (hnRNP I); polypyrimidine tract-binding protein	gi|4506243	59/65	9.2/9.3	139	EGQEDQGLTKDYGNSPLHR	61/0.00016	7
						IAIPGLAGAGNSVLLVSNLNPER	78/3.1e−07	
15	Non-POU domain-containing octamer-binding protein (NonO protein); 54 kDa nuclear RNA- and DNA-binding protein (p54/nrb)	gi|67469924	54/65	9.1/9.0	131	AAPGAEFAPNKR	28/0.41	8
						GIVEFSGKPAAR	61/0.00019	
						AVVIVDDRGRPSGK	41/0.007	
16	Same as 15	gi|67469924	54/65	9.1/8.9	188	AAPGAEFAPNKR	38/0.05	10
						LFVGNLPPDITEEEMR Oxidation (M)	40/0.016	
						FGQAATMEGIGAIGGTPPAFNR Oxidation (M)	110/1.2e−09	
18	FUS/TLS protein FUS gycline rich protein	gi|3582783	53.5/79	9.4/9.3	70	LKGEATVSFDDPPSAK	70/2e−05	3
20	Heterogeneous nuclear ribonucleo-protein M isoform a (hnRNP M)	gi|14141152	78/86	8.9/8.9	78	AFITNIPFDVK	11/16	4
						GNFGGSFAGSFGGAGGHAPGVAR	67/3.5e−05	

Table 1 continued

Spot number	Identified protein	NCBI accession number	Theor. Mr/ept. Mr (kDa)	Theor. pI/exp. pI	Mascot score[a]	Identified peptides	Ion score/expect[b]	Sequence coverage (%)
35	Lactate dehydrogenase A	gi|5031857	37/35	8.4/8.5	249	LVIITAGAR	52/0.0012	15
						DLADELALVDVIEDK	78/3.2e−06	
						GEMMDLQHGSLFLR + 2 Oxidation (M)	41/0.013	
						LKGEMMDLQHGSLFLR + 2 Oxidation (M)	68/2.8e−05	
						RVHPVSTMIK Oxidation (M)	10/23	

[a] The Mascot score is the protein score derived from the ions scores

[b] The ion score and the expectation value (the number of matches with equal or better scores that are expected to occur by chance alone) of the matched peptides are listed

modifications might be suggested. The maximum miss-cleavage of trypsin was increased to three in this search as inefficient cleavage after methylarginines was reported for trypsin. In case that the putative arginine methylation sites suggested by Mascot might be arbitrary, these sites were compared with the MeMo prediction of the protein. We listed in Table 2 the hnRNPA1 tryptic peptides containing methylarginines (at R194, 196, 206, 218, 225 and 232) suggested by both Mascot and MeMo.

3.4 Analysis of Putative Arginine Methylation of hnRNPM

We identified an RGGNRF sequence in the N-terminus (residue 56–61) of hnRNPM close to the typical RGG motif sequence. We thus expressed and purified the recombinant GST-hnRNPM (N-198) protein containing the N-terminal 198 amino acids of hnRNPM and performed in vitro methylation using the recombinant hnRNPM or a peptide KRGGNRF (corresponding to hnRNPM residue 55–61) as the methylaccepting substrate. As shown in Fig. 2, methylation of recombinant hnRNPM (N-198) can be detected when porcine brain extract S3 was used as the enzyme source. However, no methylation on this recombinant protein could be detected when HeLa cell extract or recombinant PRMT1 was used as the enzyme source. As for the hnRNPM peptide, methylation was detected by HeLa cell extract and PRMT1, but not the porcine brain extract S3 (Fig. 2). Another peptide R9 with typical RGG sequence GGRGRGGGF from human fibrillarin or FMRP protein [1] could be modified by all three enzyme sources (Fig. 2). Thus hnRNP M peptide not methylated by brain extract S3 was less likely to be due to degradation of the peptide by the brain extract since R9 could be modified by the extract. We are uncertain about the discrepancies of the in vitro methylation results among the recombinant protein and peptide. For different methylation reactions, specific cofactors might be required or inhibitors were present in different sources of enzymes or substrates.

3.5 Analysis of Putative Arginine Methylation of hnRNPI and NonO Protein

To examine the putative arginine methylation in hnRNPI and NonO protein, we isolated the proteins by immunoprecipitation and the then analyzed the precipitated proteins by immunoblotting with 7E6. The molecular massed of both proteins were close to that of immunoglobulin heavy chain. Multiple signals were detected in immunoblots for immunoprecipitates from either anti-hnRNP I or anti-NonO. Nevertheless, as shown in Fig. 3, weak but clear signals in the blots probed by 7E6 were at the same

Table 2 Putative arginine methylation sites in hnRNPA1 suggested by peptide mass fingerprint with Mascot search engine and predication by a protein arginine methylation prediction web server MeMo

Peptide sequence	Res. number	Observed mass	Delta mass	Miss cut	Modification	Predicted by MeMo
ALSKQEMASASSSQR	180–194	1,610.7440	−0.0362	1	Oxidation (M); dimethyl (R)	Y
QEMASASSSQRGRSGSGNFGGGR	184–206	2,315.0420	−0.0121	2	Oxidation (M); dimethyl (R)	Y
SGSGNFGGGRGGGFGGNDNFGR	197–218	2,058.9165	0.0040	1	2 Methyl (R)	Y
SGSGNFGGGRGGGFGGNDNFGR	197–218	2,072.9517	0.0235	1	Dimethyl (R); methyl (R)	Y
GGGFGGNDNFGRGGNFSGR	207–225	1,871.8772	0.0240	1	Dimethyl (R); methyl (R)	Y
GGGFGGNDNFGRGGNFSGRGGFGGSR	207–232	2,462.0935	−0.0159	2	Methyl (R)	Y
GGNFSGRGGFGGSR	219–232	1,340.6486	0.0031	1	2 Methyl (R)	Y

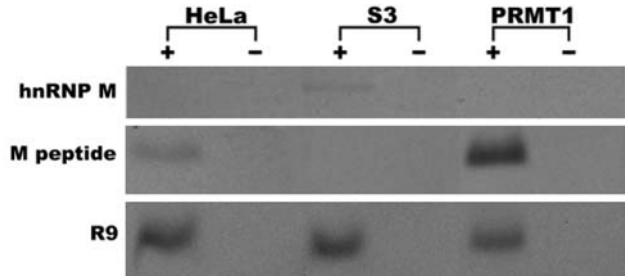

Fig. 2 In vitro methylation of the recombinant GST-hnRNPM (N-198) protein or peptide KRGGNRF as the methylaccepting substrate. In vitro methylation were performed with a final volume of 20 μL including 1.5 μCi [*methyl*-^{3}H]-AdoMet, the enzyme source and the substrates. HeLa cell extract protein (30 μg), porcine brain extract S3 (20 μg) or recombinant GST-PRMT1 were used as the enzyme source. GST-hnRNP M (N-198) containing the N-terminal 198 amino acids of hnRNPM (2 μg), the hnRNP M peptide KRGGNRF (1 μg), or the R9 peptide GRGGRGGGF (1 μg) were included as the methylaccepting substrates

position of hnRNPI or NonO protein when the same blot was re-probed with anti-hnRNPI or anti-NonO antibodies. The signals were present in the input lanes but not in a blank IP controls. It is to be noted that the proteins in the IP lanes ran slightly slower than those in the input lanes. The results indicate the presence of methylarginines in hnRNPI and NonO protein.

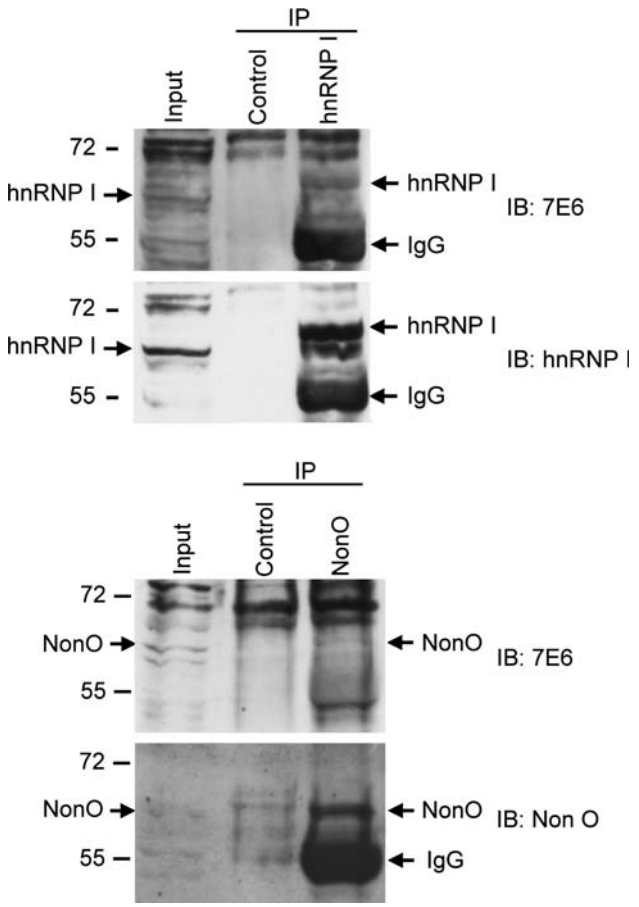

Fig. 3 Detection of in vivo methylation of hnRNPI and NonO protein. By immunoprecipitation and immunoblotting. HeLa cell extract was immunoprecipitated with antibodies against hnRNPI or NonO as described in Sect. 2. The immunoprecipitants were separated by SDS–PAGE (10% resolving gel) and submitted to immunoblot analysis with the methylarginine-specific antibody 7E6. After the first detection, the blot was stripped of bound antibodies and reprobed with the antibody used for immunoprecipitation

4 Discussion

As arginine methylation play important roles in transcriptional regulation, signal transduction, RNA splicing, protein transport, and embryogenesis [2, 25], identification of the complete methylarginine-containing proteome and development of a fast and robust protocol to examine protein arginine methylation should be critical. In this study we successfully identified methylarginine-containing proteins in HeLa cells by comparing the 2-DE patterns of protein stains with immunoblots of a methylarginine-specific antibody. As we conducted first dimensional IEF electrophoresis using 7-cm IPG strips, limited separation was inevitable. However, it made the following immunoblotting of the 2-DE gels rather feasible compared to the blotting of 2-DE gels from standard 18-cm strips. The whole process is fast and can be completed in 2 days and thus can be used for routine applications.

RNA binding proteins including hnRNPA1, hnRNPA2/B1, FUS, hnRNPG and Sam 68 with verified methylarginine residues were identified, confirming the effectiveness of this approach. HnRNPA1 was recognized as a methylarginine-containing protein and was used to illustrate the type I protein arginine methyltransferase activity about 20 years ago [15, 30]. FUS/TLS has also been shown to contain more than 20 arginine methylation sites [24, 27]. Arginine methylation of Sam68 by PRMT1 in vivo [6] as well as specific arginine methylation sites in this protein have been demonstrated [24, 27]. These proteins have been identified or suggested to be methylated by PRMT1, the predominant type I arginine methyltransferase. As the 7E6 antibody recognize MMA or aDMA formed by the type I arginine methyltransferase, other putative methylarginine-containing proteins identified are most likely to be substrates of the type I enzyme.

We also indicate that putative methylarginine sites can be suggested through detailed peptide mass fingerprint analyses (Table 2) in combination with an online prediction program MeMo. Six putative arginine methylation sites including R194, 196, 206, 218, 225 and 232 of hnRNPA1 can be predicted. Four methylarginine sites (R194, 206, 218 and 225) have been reported previously [15, 24, 30], indicating the feasibility of the approach. Nevertheless, confirmation of the two putative novel arginines methylation sites (R196, R232) requires further supports such as MS/MS sequencing data.

We identified three nucleic acid binding proteins hnRNPM, hnRNPI and the NonO protein as potential methylarginine-containing proteins. General analysis of arginine methylation in hnRNP complex showed that hnRNPM and hnRNPI were not in vivo methylated but was weakly methylated in vitro [22]. On the contrary, hnRNPA1, A2/B1 and G along with a few other hnRNP proteins were clearly detected by in vivo as well as in vitro methylation. In this study we showed that recombinant GST- hnRNPM (N198) fusion protein could be in vitro methylated under specific conditions. The RGGNRF sequence at residue 56–61 is likely to be the methylation site as recombinant PRMT1 and HeLa cell extract can methylate the KRGGNRF peptide in vitro. Immunoprecipitated hnRNPI and NonO protein from HeLa cells can be further detected by Western blot with methylarginine-specific antibody, suggesting the modification of these proteins.

It is likely that the identified RNA binding proteins are more abundant than other methylarginine-containing proteins not detected by the Coomassie blue stain, thus were analyzed and identified. Some of the spots like 2, 3, 4 and 35 correspond to heavily stained protein spots. Four highly abundant metabolic enzymes were identified for these spots. Malate dehydrogenase, hnRNPA2/B1 and glyceraldehydes 3-phosphate dehydrogenase were all identified for spot 3 with ion scores higher than those indicating identity. It is likely that hnRNPA2/B1 is the target for 7E6 in spot 3 while the other two abundant enzymes could not be separated. On the other hand, Fackelmayer proposed that arginine methylation might be a protection mechanism of the arginine residues from the attack of reactive dicarbonyl agents produced as by-products of natural metabolic pathways [7]. Whether sublevel of the abundant proteins was modified and thus detected requires more experimental evidences.

In conclusion, we demonstrated that separation by 2-DE, detection by western blot with modification-specific antibodies, and identification of signals by mass spectrometry can be easily conducted and applied generally to all PTM. Protein detection with other MS-compatible and more sensitive staining methods such as Sypro Ruby than Coomassie blue should help to identify less abundant modified proteins. Elimination of highly abundant proteins that might mask the less abundant modified proteins prior to the 2-DE separation will increase the opportunities identifying the targets.

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