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Proteomic Analysis of Stable Protein Methylation in Lymphoblastoid Cells[1]

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We investigated the global distribution of methylaccepting proteins in lymphoblastoid cells by two-dimensional (2-D) gel electrophoresis. The 2-D electrophoreograms of normal and hypo-methylation (cells grown with a methyltransferase inhibitor adenosine dialdehyde) protein extracts did not exhibit significant differences. However, *in vitro* methylation of the hypomethylated extracts in the presence of the methyl-group donor *S*-adenosyl-[methyl-^{3}H]-methionine revealed close to a hundred signals. Less than one-fifth of the signals could be correlated with protein stains, indicating that most of the methylaccepting proteins are low abundant ones. We analyzed six of the spots that can be correlated with protein stains and suggested their identities. Among these putative protein methylacceptors, three are heterogeneous nuclear ribonucleoproteins (hnRNPA2/B1 and hnRNP K) that are reportedly methylated in their arginine- and glycine-rich RGG motifs.

Key words: hnRNPA2/B1, posttranslational modification, two-dimensional gel electrophoresis.

One of the major challenges in the post-genomic era is to investigate the expression of the complete set of cellular proteins (*1*). Posttranslational modifications increase the complexity of proteins beyond the combination of twenty amino acids. Protein *N*-arginine methylation is an irreversible modification of the guanidino nitrogens of arginyl residues that accounts for the majority of stable cellular protein methylation (*2, 3*). Most methylarginines identified on proteins appear to be N^G-monomethylarginine (MMA) and asymmetric N^G,N^G-dimethylarginines (aDMA) that occur among RNA binding proteins within the Arg-Gly-Gly context (*2–4*). These modifications were catalyzed by type I arginine methyltransferases (*5*). Type II methyltransferases modify proteins such as myelin basic protein (*6*), the core snRNP SmD1, D3 (*7*), B/B′, and one of the Sm-like proteins, LSm4 (*8*). The methylation products are MMA and symmetric $N^G,N^{G'}$-dimethylarginine (sDMA).

Protein arginine methylation has been proposed to be involved in various cellular processes. Firstly, arginine methylation was suggested to be important for RNA binding, and methylated recombinant heterogeneous nuclear ribonucleoproteins A1 (hnRNP A1) showed reduced RNA binding activity as compared to the unmethylated form (*9*). However, methylation of Hrp1p, a yeast hnRNP, did not affect its specific RNA binding activity (*10*). Secondly, since the predominant protein arginine methyltransferase PRMT1 interacts with proteins involved in signal transduction pathways such as TIS 21 (*11*), the intracellular domain of the interferone α/β receptor (*12*), and ILF3 (*13*), protein arginine methylation was suggested to play roles in signal transduction (*11, 14*). Recently, arginine methylation of certain proteins such as STAT1 was shown to directly affect their binding with other proteins (*15*). The modification may also modulate protein-protein interactions by interfering with other important modifications of neighboring motifs of the same protein, as reported for Np13p (*16*), Sam 68 (*17*), and histone H4 (*18*). Lastly, arginine methylation has been implicated in subcellular localization. Yeast deficient in RMT1/HMT1, a predominant arginine methyltransferase, has problems in hnRNP and mRNA nuclear export (*19*). Furthermore, decreased methylation of hnRNP A2/B1 resulted in accumulation of the protein in the nucleus rather than in the cytoplasm (*20*).

In view of that this type of posttranslational modification plays important roles in cells, identification of arginine methylaccepting proteins ought to be an important task. Arginine methylaccepting substrates have been identified and studied through different approaches. Proteins such as fibrillarin (*21*), nucleolin (*22*), and hnRNPA1 (*23*) were found to contain asymmetric dimethylarginines on direct biochemical analyses. Other proteins with similar arginine-

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Abbreviations: aDMA, asymmetric N^G, N^G-dimethylarginine; sDMA, symmetric N^G, $N^{G'}$-dimethylarginine; MMA, N^G-monomethylarginine; AdOx, adenosine dialdehyde; RMT, arginine methyltransferase; PRMT, protein arginine methyltransferase; GST, glutathion-S-transferase; AdoMet, *S*-adenosylmethionine; hnRNP, heterogeneous nuclear ribonucleoproteins; RGG box, arginine and glycine rich motif, MALDI-TOF, matrix assisted laser desorption ionization-time of flight; 2-D, two-dimensional; IPG, immobilized pH gradient.

and glycine-rich motifs, mostly RNA binding proteins such as herpes simplex virus ICP27 protein (*24*), FGF2 (*25*), and EWS (*26*), have also been identified as methylaccepting substrates. Recently, proteins without typical arginine- and glycine-rich sequences have been found to contain methylarginines. The methyl group on the PABPII protein was found on a RXR motif (*27*), while the methylarginine in STAT1 is located in the EIRQY context (*15*).

Alternatively, cells were made deficient in methyltransferase activity by either genetic approaches (*28, 29*) or by chemical treatment (*3*). The methylaccepting sites would not be occupied *in vivo*, and can be detected by further *in vitro* methylation reactions and analyzed by gel electrophoresis. Although previous studies had involved two-dimensional (2-D) gel electrophoresis for product analyses, the methylated proteins revealed on fluorography could not be identified unambiguously. Candidate proteins were only inferred from their molecular masses and pIs (*3, 29, 30*).

In this study, we treated lymphoblastoid cells with an indirect methyltransferase inhibitor, adenosine dialdehyde (AdOx). After *in vivo* or *in vitro* methylation reactions, cell extracts were separated by 2-D electrophoresis. The proteins of interest were excised from the gels, digested with a specific protease and then subjected to MALDI (matrix assisted laser desorption ionization)-TOF (time of flight) mass spectroscopy for protein identification. With this approach, we have confirmed some previously known methylaccepting proteins and identified some novel putative ones.

MATERIALS AND METHODS

Cell Cultures and Protein Extraction—EB-virus transformed lymphoblastoid cell line 003 was grown as previously described (*2*). For methylation reactions, cells were grown in the presence of 20 μM AdOx for 72 h for the accumulation of methylaccepting proteins. Cells collected from a 75 cm^2 culture flask were resuspended in 600 μl of 2-D extraction buffer (100 mM ammonium carbonate, pH 8.0, 0.5 mM PMSF, 2 mM EDTA, 2% NP-40). The cells were incubated on ice for 10 min and then lysed by sonication. Cell debris was removed by centrifugation at 12,000 ×*g* for 20 min. Proteins in the extracts were quantified by means of the BCA assay (Pierce) with bovine serum albumin as the standard. Extracts thus prepared gave poor methylaccepting signals after the *in vitro* methylation reaction. We thus followed the protocol of Li *et al.* (*2*) to prepare the extracts for the methylation reaction using extraction buffer (phosphate-buffered saline [10 mM dibasic sodium phosphate, 1.8 mM monobasic potassium phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4], 5% glycerol, 1 mM disodium EDTA, 1 mM EGTA, 40 μg/ml leupeptin and aprotinin, 20 μg/ml pepstatin, 1 mM PMSF, 0.5% Triton X-100).

In Vitro Methylation Reaction and Fluorography—Total extracts (100 μg proteins) were incubated with 10 μCi of [*methyl*-^{3}H]-AdoMet (60 Ci/mmol, Amersham) in the presence or absence of recombinant yeast GST-RMT1 arginine methyltransferase in methylation reaction buffer (50 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM EGTA). The final volume was 70 μl and the reaction was carried out for 2 h at 30°C. The reaction was stopped by incubation on ice, followed by dialysis using a mini dialysis unit (MW cut off 3,500; Pierce) for salt removal. Recombinant yeast GST-RMT methyltransferase was prepared as previously described (*31*).

Two-Dimensional Gel Electrophoresis—Samples (1 mg) were loaded onto immobilized pH gradient (IPG) strips (pH 3–10, 13 cm) through overnight rehydration at room temperature in a reswelling tray. The samples were prepared by lyophilization and then resuspension in 250 μl of 8 M urea, 2% (w/v) CHAPS, 2% IPG buffer, and 2.8 mg/ml dithiothreitol. Isoelectrophocusing (IEF) was carried out in a Multiphor II system as instructed by the manufacturer (Amersham Biosciences). Upon completion of IEF (17,000 Volt-hours), the IPG strips were equilibrated in 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% (w/v) SDS, 15.4 mg/ml dithiothreitol, and 0.0125% bromophenol blue for 15 min. The strips were then stored frozen at –80°C or used immediately. Gradient SDS polyacrylamide gels (5–20%, 17 × 20 × 1 mm) were employed for the second dimensional separation in a Bio-Rad PROTEAN II xi system. Proteins were visualized by colloidal blue staining for 2 days and destaining was carried out in 25% methanol (*32*). Z3 (Compugen) and Melanie 3 (Genebio) software was used to analyze the gel images. Gels containing isotopically labeled proteins were treated with EN^{3}HANCE (Du Pont NEN), dried and then exposed to X-ray film (Kodak, MS) at –75°C. Since silver-stained gels gave poor fluorographic results and large gels tended to crack after drying, we used colloidal blue staining and 13 cm IPG strips for the analysis of radioactive samples.

In Gel Protein Digestion—Protein spots were excised from the polyacrylamide gel with pipette tips, and the gel plugs were washed extensively in 10% acetic acid and 50% methanol in water. They were then incubated in 200 mM Tris, pH 8.8, 50% acetonitrile for 30 min. The buffer was removed and the gel plugs were dehydrated by adding acetonitrile, followed by vacuum centrifugation. Lys-C protease solution (Wako, 0.033 mg/ml in 100 mM ammonium bicarbonate buffer, pH 9.2) or trypsin solution (Promega, 0.033 mg/ml in 50 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$) was then added to restore the gel plugs to their original volume. Digestion was carried out at 42 and 25°C overnight for Lys-C and trypsin proteases, respectively. The digests were then acidified by adding 2% TFA. Peptides were extracted from the gel plugs sequentially with 0.1% TFA, in water, 30% acetonitrile in water with 0.1% TFA, and finally 60% acetonitrile in water and 0.1% TFA. The solutions were combined, dried to less than 50 μl, and then desalted with a Ziptip (Millipore) pipette tip following the manufacturer's instructions.

MALDI-TOF MS Analysis—Peptide mass mapping was performed on a Bruker (Bruker-Daltonics, Bremen, Germany) REFLEX III time-of-flight mass spectrometer equipped with a SCOUT source and delayed extraction. Detection was performed in the positive ion reflector mode with each mass determination being the average of 100 spectra. Samples for mass measurement were prepared using the solution-phase nitrocellulose method (*33*) with α-cyano-4-hydroxycinnamic acid as the matrix. Alternatively, samples were deposited on a Bruker 600 micron AnchorChip according to the manufacturer's instructions. Angiotensin II (1,045.54 amu), adrenocorticotropic hormone fragment 18–39 (2,465.73 amu), and somatostatin (3,146.47) were used as external calibration standards. The peptide mass spectra obtained on MALDI-TOF analysis were ana-

lyzed using PeptidIdent (http://tw.expasy.org) and Profound (http://129.85.19.192/profound/).

RESULTS AND DISCUSSION

Stable Methylaccepting Proteins in Lymphoblastoid Total Extracts Analyzed by 2-D Electrophoresis—Arginine methylation is a stable posttranslational modification different from protein phosphorylation that is regulated reversibly by kinases and phosphatases. Even though six different protein arginine methyltransferases have been identified so far (*11, 34–39*), no demethylase activity has been reported. Therefore, once an arginine methylaccepting protein is methylated, the methyl group will stay on the protein. Proteomic analysis of protein phosphorylation benefits by the comparison of phophatase-treated or untreated samples (*40, 41*). In this study we used a methyltransferase inhibitor, AdOx, for the accumulation of proteins in the hypomethylation state. Extracts of AdOx-treated or -untreated lymphoblastoid cells were prepared and isolated on pH 3–10 IPG strips. The proteins were further separated in the second dimension by SDS-PAGE (8–15% gradient gel) and visualized by colloidal blue staining (Fig. 1). Typically, we were able to detect more than 1,000 spots on a 13-cm IPG strip loaded with 1 mg of protein. However, no consistent significant differences between the 2D patterns of the AdOx-treated and -untreated cells could be found using either the Z3 or Melanie 3 program. Even though AdOx treatment can cause the accumulation of methylaccepting sites in cellular proteins that can be methylated subsequently *in vitro* (*2, 4*), these sites available upon treatment (after confluency) are likely to comprise only a minor fraction of all the methylaccepting sites in proteins. It is also possible that arginine methylation does not significantly change the overall charge property or molecular masses of the proteins to be detected by 2-D electrophoresis.

We prepared total extracts of hypomethylated lymphoblastoid cells for *in vitro* methylation reactions in the presence of the radioactive methyl-group donor [*methyl*-^{3}H]-AdoMet in the presence of exogenous recombinant yeast GST-RMT1 enzyme. Since six protein arginine methyltransferases have been identified in mammalian systems and are likely to be regulated differently (*11, 34–39*), we used the recombinant yeast enzyme, which is stables and has a broader substrate specificity than the mammalian enzyme (*31*). The reaction products were then separated by two-dimensional gel electrophoresis and the methylated proteins were visualized by fluorography. As shown in Fig. 2, more than a hundred radioactive spots representing putative methylaccepting proteins were detected with our 2-D gel system. The addition of GST-RMT1 increased the intensity of some spots but few extra spots were detected (data not shown), indicating the exogenous enzyme recognized similar methylaccepting substrates to those recognized by the endogenous enzyme, as shown previously on SDS-PAGE analyses (*2*). Of the more than one hundred putative methylaccepting proteins revealed by two-dimensional gel electrophoresis of *in vitro* radio-labeled lymphoblastoid total extracts, less than one-fifth could be correlated with protein stains. The results indicate that most

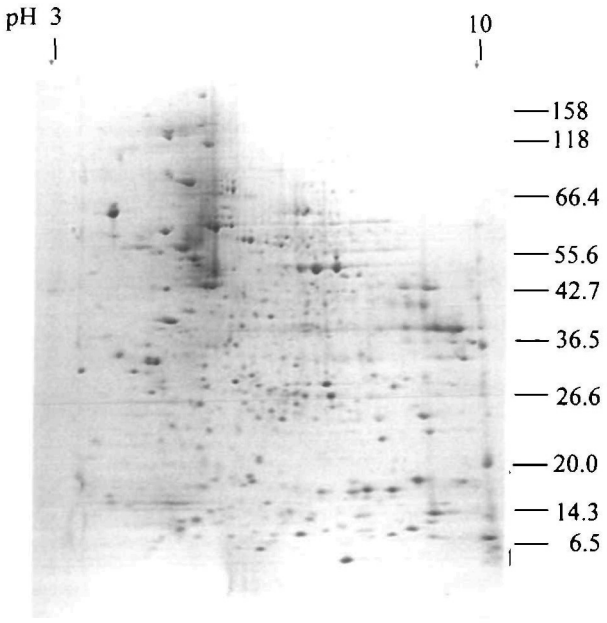

Fig. 1. **2-DE pattern of a lymphoblastoid total extract.** Lymphoblastoid total extracts (1 mg protein) prepared from AdOx-treated cells were analyzed by 2-DE and stained with colloidal blue as described.

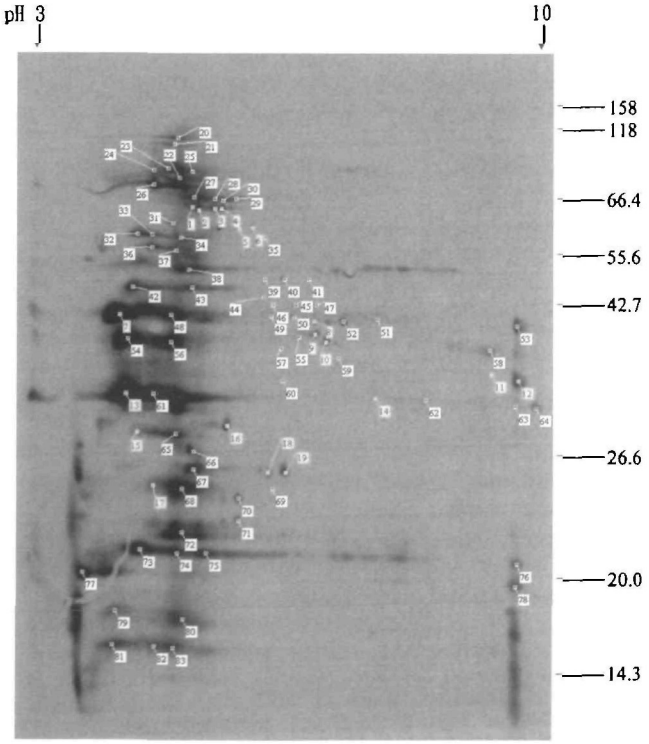

Fig. 2. **Protein methylacceptors revealed after *in vitro* methylation and 2-DE analyses.** Lymphoblastoid total extracts (200 μg) were incubated with exogenous recombinant yeast RMT1 methyltransferase and [*methyl*-^{3}H]-AdoMet. The reaction mixtures were then dialyzed with a mini dialysis unit, lyophilized and subjected to 2-DE analysis. The gel was stained with colloidal blue, soaked in ENHANCER and then dried for fluorography. The figure represents 2-week exposure. Spots 1–19 are the radioactive spots that can be matched with protein stains.

of the methylaccepting proteins are low abundant ones that are difficult to detect on colloidal staining. This can also help to explain why the AdOx treatment did not result in different protein patterns on 2-D gel electrophoresis.

Many of the reported arginine methyl-accepting proteins (*e.g.* fibrillarin, hnRNPs, histones and Sm proteins, see Table I) have high pI values that are beyond the resolution of our IEF gel (pH 3–10). We observed more than ten radioactive spots at the basic limit of the 2-D gel corresponding to molecular masses ranging from 40 to 14 kDa. These signals are likely to be due to proteins. Interestingly, the strongest signals on the 2-D gel came from the region between pH 4–6, differing from the pIs of most reported arginine methylaccepting proteins (Table I).

Identification of the Methylaccepting Proteins—To identify the methylaccepting proteins, we increased the loading of the methylated samples for 2-D gel electrophoresis. However, most of the heavily methylated signals could not be matched back to dye-stained spots. The results indicate that most of the methylaccepting proteins are low abundant ones that are difficult to detect on colloidal staining, and thus help to explain why the AdOx treatment did not result in different protein patterns on 2-D gel electrophoresis.

We were able to correlate about 20 radioactive signals with corresponding dye-stained protein spots. These spots were excised, protease-digested, and then subjected to MALDI-TOF analyses. We were able to identify six of the putative methylaccepting proteins using the peptide masses along with the pIs and molecular masses for the protein spots (Table II). Among these proteins, hnRNPA2/B1 and hnRNP K were known methylaccepting proteins containing RGG motifs (*20, 42*). It is likely that these proteins are more abundant ones than other methylaccepting proteins, and thus can be detected and analyzed by means of our approach.

For the other three putative novel methylacceptors, further experiments failed to show that protein disulfide isomerase (PDI) was arginine methylated. The recombinant yeast RMT enzyme failed to methylate bovine PDI (Calbiochem). This protein was not recognized by the mono- and di-methylarginine Ab (data not shown). To determine whether PDI is modified through another type of protein methylation or just has the same molecular weight and pI as another low abundant methylaccepting protein needs more experimental work. Whether or not prohibitin and proteasome subunit alpha type 5 are real methylacceptors is also under investigation.

Identification of the Methylaccepting Sites in the hnRNPA2/B1 Proteins—We detected the specific presence of hnRNPA2/B1 in the immunoprecipitants with a methylarginine-specific antibody (data not shown), further confirming the arginine methylation of the proteins. The difference between hnRNP A2 and B1 is the additional twelve amino acid residues at the N-terminus of B1. According to previous reports, we designated the more acidic one A2. We analyzed both the A2 and B1 spots using AdOx-treated or -untreated samples. Interestingly, as shown in Fig. 3, two extra peaks at 2,386.6 and 2,400.7 *m/z*, and a higher one at 2,509.6 *m/z* were observed for the trypsin digests of the B1 spots for extracts without AdOx treatment (theoretically in the normal methylation state), but not for those of the corresponding spots for AdOx-treated extracts (theoretically in the hypomethylation state). Although no peptide could be matched with these molecular masses on PeptIden analysis, mono- or di-methyl methylation of peptides containing RG-rich sequences was suggested by further FINDMOD analyses (http://tw.expasy.org/tools/findmod/; *47*). The peptide comprising residues 204 to 228 (GGNFGFGDS**R**GGGGNFGPGPGSNFR, mass 2,372.055) with one trypsin miss cleavage and mono or dimethylarginine at Arg-213 would well explain the extra two masses. It is possible that the higher signal, 2,509.6, could be due to monoarginine methylation at residue Arg-266 in the peptide covering residues 239–266 (GFGDGYNGYGGGPGGGNFGGSPGYGGG**R**, mass 2,495.039). We obtained similar results with the hnRNPA2 sample (data not shown).

Nichols *et al.* (*20*) showed that the methylation state of RGG in hnRNP A2 might interfere with its subcellular localization. The addition of AdOx (low methylation) shifted the hnRNPA2 protein to the cytoplasm and then the nucleus. Removal of residues R203 to G265 reduced the methylation level of the recombinant protein to only 5%,

TABLE I. **List of known arginine methylaccepting proteins.**

Protein	SWISS PROT No.	MW	PI	References
Fibrillarin	P22087	33,784.22	10.18	*21*
Nucleolin	P19338	76,212.88	4.59	*22*
HnRNP				*41*
hnRNPA1	P09651	38,714.59	9.26	*23*
hnRNPA2/B1	P22626	37,429.70	8.97	*20*
(A2)		36,005.98	8.67	
E1B-AP5	O76022	95,809.60	6.49	*43*
Histone				
H2A	P28001	14,004.30	11.05	*36*
H3	P16106	15,272.89	11.13	*44, 45*
H4	P02304	11,236.15	11.36	*18, 46*
polyA binding protein II (PABP2)	O43484	32,749.07	5.04	*27*
ILF3 (NFAR2)	Q9BZH4	95,591.74	8.88	*13*
Sam68	Q07666	48,227.34	8.73	*17*
	Q99760	44,027.41	6.78	
EWS	Q01844	68,478.24	9.37	*26*
STAT1	P42224	87,334.76	5.74	*15*
MBP	P02686	33,117.10	9.79	*6*
SmD1	P13641	13,281.57	11.56	*7*
SmD3	P43331	13,916.25	10.33	*7*

TABLE II. **List of putative protein methylacceptors identified in this study.**

Spot ID	SWISS-PROT Accession No.	Protein name	Measured pI/MW	Theoretical pI/MW	Peptide matches/ detected peptides	PeptIden score	Profound score	Sequence coverage (%)
2	Q07244	hnRNPK	5.2/60	5.39/50.98	9/14	0.57	0.62	22
6	P30101	Protein disulfide isomerase	6.0/57	5.60/54.26	11/14	0.79	0.85	31
11	P22626	hnRNPA2	8.5/36.5	8.67/36.01	7/11	0.64	2.38	32
12	P22626	hnRNPB1	8.9/36.5	8.97/37.43	7/10	0.70	2.43	32
15	P28066	Proteasome subunit alpha type 5	4.5/27	4.69/26.47	6/10	0.40	2.43	27
16	P35232	Prohibitin	5.8/29	5.57/29.79	8/14	0.57	1.77	37

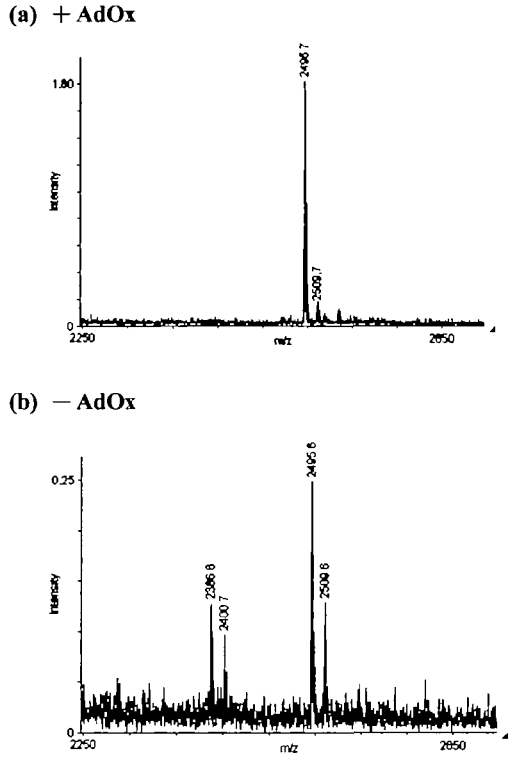

Fig. 3. **MALDI-TOF spectrum of tryptic peptide fragments derived from hnRNPB1.** (a) The hnRNPB1 spot was from 2-D gels used to separate extracts prepared from lymphoblastoid cells treated with AdOx (+AdOx). (b) The hnRNPB1 spot was from 2-D gels used to separate extracts prepared from lymphoblastoid cells not treated with AdOx (–AdOx).

indicating the major methylation site is within the deleted segment. However, the R203 residue in a peptide fragment (194–205) of hnRNPA2/B1 purified from a human tumor cell line was not methylated, as determined on peptide sequencing (*48*). Thus, our results are the first providing chemical evidence suggesting putative methylaccepting sites of hnRNPA2/B1 at Arg-213 and Arg-226.

To determine whether or not the other three putative methylaccepting proteins, protein disulfide isomerase, prohibitin and proteosome subunit alpha type 5, we identified are methylacceptors requires further investigation. Prohibitin had been reported to be post-translationally modified in younger but not older cells, but no positive results were obtained for prohibitin phosphorylation (*49*). Whether or not prohibitin can be modified by methylation is an interesting question. Since AdOx can inhibit the activity of all methyltransferases using S-adenosylmethionine as the methyl group donor, other types of protein methylation besides arginine methylation could also be revealed by this approach. Thus, for putative novel methylaccpetor proteins, more analyses are required. As for low abundant methylaccepting proteins, previous fractionation or concentration procedures should be included before the 2-D analyses for a better chance of detection.

REFERENCES

1. Pandey, A. and Mann, M. (2000) Proteomics to study genes and genomes. *Nature* **405**, 837–846
2. Li, C., Ai, L.S., Lin, C.H., Hsieh, M., Li, Y.C., and Li, S.Y. (1998) Protein N-arginine methylation in adenosine dialdehyde-treated lymphoblastoid cells. *Arch. Biochem. Biophys.* **351**, 53–59
3. Najbauer, J., Johnson, B.A., Young, A.L., and Aswad, D.W. (1993) Peptides with sequences similar to glycine, arginine-rich motifs in proteins interacting with RNA are efficiently recognized by methyltransferase(s) modifying arginine in numerous proteins. *J. Biol. Chem.* **268**, 10501–10509
4. Lin, C.H., Hsieh, M., Li, Y.C., Li, S.Y., Pearson, D.L., Pollard, K.M., and Li, C. (2000) Protein N-arginine methylation in subcellular fractions of lymphoblastoid cells. *J. Biochem.* **128**, 493–498
5. Gary, J.D. and Clarke, S. (1998) RNA and protein interactions modulated by protein arginine methylation. *Prog. Nucleic Acid Res. Mol. Biol.* **61**, 65–131
6. Baldwin, G.S. and Carnegie, P.R. (1971) Specific enzymatic methylation of an arginine in the experimental allergic encephalomyelitis protein from human myelin. *Science* **171**, 579–581
7. Brahms, H., Raymackers, J., Union, A., de Keyser, F., Meheus, L., and Luhrmann, R. (2000) The C-terminal RG dipeptide repeats of the spliceosomal Sm proteins D1 and D3 contain symmetrical dimethylarginines, which form a major B-cell epitope for anti-Sm autoantibodies. *J. Biol. Chem.* **275**, 17122–17129
8. Brahms, H., Meheus, L., de Brabandere, V., Fischer, U., and Luhrmann, R. (2001) Symmetrical dimethylation of arginine residues in spliceosomal Sm protein B/B′ and the Sm-like protein LSm4, and their interaction with the SMN protein. *RNA* **7**, 1531–1542
9. Rajpurohit, R., Paik, W.K., and Kim, S. (1994) Effect of enzymatic methylation of heterogeneous ribonucleoprotein particle A1 on its nucleic-acid binding and controlled proteolysis. *Biochem. J.* **304**, 903–909
10. Valentini, S.R., Weiss, V.H., and Silver, P.A. (1999) Arginine methylation and binding of Hrp1p to the efficiency element for mRNA 3′-end formation. *RNA* **5**, 272–280
11. Lin, W.J., Gary, J.D., Yang, M.C., Clarke, S., and Herschman, H.R. (1996) The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine N-methyltransferase. *J. Biol. Chem.* **271**, 15034–15044
12. Abramovich, C., Yakobson, B., Chebath, J., and Revel, M.A. (1997) protein-arginine methyltransferase binds to the intracytoplasmic domain of the IFNAR1 chain in the type I interferon receptor. *EMBO J.* **16**, 260–266
13. Tang, J., Kao, P.N., and Herschman, H.R. (2000) Protein-arginine methyltransferase I, the predominant protein-arginine methyltransferase in cells, interacts with and is regulated by interleukin enhancer-binding factor 3. *J. Biol. Chem.* **275**, 19866–19876
14. Aletta, J.M., Cimato, T.R., and Ettinger, M.J. (1998) Protein methylation: a signal event in post-translational modification. *Trends Biochem. Sci.* **23**, 89–91
15. Mowen, K.A., Tang, J., Zhu, W., Schurter, B.T., Shuai, K., Herschman, H.R., and David, M. (2001) Arginine methylation of STAT1 modulates IFNalpha/beta-induced transcription. *Cell* **104**, 731–741
16. Yun, C.Y. and Fu, X.D. (2000) Conserved SR protein kinase functions in nuclear import and its action is counteracted by arginine methylation in *Saccharomyces cerevisiae*. *J. Cell Biol.* **150**, 707–718
17. Bedford, M.T., Frankel, A., Yaffe, M.B., Clarke, S., Leder, P., and Richard, S. (2000) Arginine methylation inhibits the binding of proline-rich ligands to Src homology 3, but not WW, domains. *J. Biol. Chem.* **275**, 16030–16036
18. Wang, H., Huang, Z.Q., Xia, L., Feng, Q., Erdjument-Bromage, H., Strahl, B.D., Briggs, S.D., Allis, C.D., Wong, J., Tempst, P., and Zhang, Y. (2001) Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. *Science* **293**, 853–857
19. Shen, E.C., Henry. M.F., Weiss, V.H., Valentini, S.R., Silver,

P.A., and Lee, M.S. (1998) Arginine methylation facilitates the nuclear export of hnRNP proteins. *Genes Dev.* **12**, 679–691
20. Nichols, R.C., Wang, X.W., Tang, J., Hamilton, B.J., High, F.A., Herschman, H.R., and Rigby, W.F. (2000) The RGG domain in hnRNP A2 affects subcellular localization. *Exp. Cell Res.* **256**, 522–532
21. Lischwe, M.A., Ochs, R.L., Reddy, R., Cook, R.G., Yeoman, L.C., Tan, E.M., Reichlin, M., and Busch, H. (1985) Purification and partial characterization of a nucleolar scleroderma antigen (Mr = 34,000; pI, 8.5) rich in NG,NG-dimethylarginine. *J. Biol. Chem.* **260**, 14304–14310
22. Lischwe, M.A., Cook, R.G., Ahn, Y.S., Yeoman, L.C., and Busch, H. (1985) Clustering of glycine and NG,NG-dimethylarginine in nucleolar protein C23. *Biochemistry.* **24**, 6025–6028
23. Rajpurohit, R., Lee, S.O., Park, J.O., Paik, W.K., and Kim, S. (1994) Enzymatic methylation of recombinant heterogeneous nuclear RNP protein A1. Dual substrate specificity for S-adenosylmethionine:histone-arginine N-methyltransferase. *J. Biol. Chem.* **269**, 1075–1082
24. Mears, W.E. and Rice, S.A. (1996) The RGG box motif of the herpes simplex virus ICP27 protein mediates an RNA-binding activity and determines *in vivo* methylation. *J. Virol.* **70**, 7445–7453
25. Klein, S., Carroll, J.A., Chen, Y., Henry, M.F., Henry, P.A., Ortonowski, I.E., Pintucci, G., Beavis, R.C., Burgess, W.H., and Rifkin, D.B. (2000) Biochemical analysis of the arginine methylation of high molecular weight fibroblast growth factor-2. *J. Biol. Chem.* **275**, 3150–3157
26. Belyanskaya, L.L., Gehrig, P.M., and Gehring, H. (2001) Exposure on cell surface and extensive arginine methylation of ewing sarcoma (EWS) protein. *J. Biol. Chem.* **276**, 18681–18687
27. Smith, J.J., Rucknagel, K.P., Schierhorn, A., Tang, J., Nemeth, A., Linder, M., Herschman, H.R., and Wahle, E. (1999) Unusual sites of arginine methylation in Poly(A)-binding protein II and *in vitro* methylation by protein arginine methyltransferases PRMT1 and PRMT3. *J. Biol. Chem.* **274**, 13229-13234
28. Pawlak, M.R., Scherer, C.A., Chen, J., Roshon, M.J., and Ruley, H.E. (2000) Arginine N-methyltransferase 1 is required for early postimplantation mouse development, but cells deficient in the enzyme are viable. *Mol. Cell Biol.* **20**, 4859–4869
29. Frankel, A. and Clarke, S. (1999) RNase treatment of yeast and mammalian cell extracts affects *in vitro* substrate methylation by type I protein arginine N-methyltransferases. *Biochem. Biophys. Res. Commun.* **259**, 391–400
30. Najbauer, J. and Aswad, D.W. (1990) Diversity of methyl acceptor proteins in rat pheochromocytoma (PC12) cells revealed after treatment with adenosine dialdehyde. *J. Biol. Chem.* **265**, 12717–12721
31. Gary, J.D., Lin, W.J., Yang, M.C., Herschman, H.R., and Clarke, S. (1996) The predominant protein-arginine methyltransferase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **271**, 12585–12594
32. Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. (1988) *Electrophoresis* **9**, 255–262.
33. Landry, F., Lombardo, C.R., and Smith, J.W. (2000) A method for application of samples to matrix-assisted laser desorption ionization time-of-flight targets that enhances peptide detection. *Anal. Biochem.* **279**, 1–8
34. Scott, H.S., Antonarakis, S.E., Lalioti, M.D., Rossier, C., Silver, P.A., and Henry, M.F. (1998) Identification and characterization of two putative human arginine methyltransferases (HRMT1L1 and HRMT1L2). *Genomics* **48**, 330–340
35. Tang, J., Gary, J.D., Clarke, S., and Herschman, H.R. (1998) PRMT 3, a type I protein arginine N-methyltransferase that differs from PRMT1 in its oligomerization, subcellular localization, substrate specificity, and regulation. *J. Biol. Chem.* **273**, 16935–16945
36. Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W., and Stallcup, M.R. (1999) Regulation of transcription by a protein methyltransferase. *Science* **284**, 2174–2177
37. Rho, J., Choi, S., Seong, Y.R., Cho, W.K., Kim, S.H., and Im, D.S. (2001) Prmt5, which forms distinct homo-oligomers, is a member of the protein-arginine methyltransferase family. *J. Biol. Chem.* **276**, 11393–11401
38. Branscombe, T.L., Frankel, A., Lee, J.H., Cook, J.R., Yang, Z., Pestka, S., and Clarke, S. (2001) PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins. *J. Biol. Chem.* **276**, 32971–32976
39. Frankel, A., Yadav, N., Lee, J., Branscombe, T.L., Clarke, S., and Bedford, M.T. (2002) The novel human protein arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. *J. Biol. Chem.* **277**, 3537–3543
40. Kaufmann, H., Bailey, J.E., and Fussenegger, M. (2001) Use of antibodies for detection of phosphorylated proteins separated by two-dimensional gel electrophoresis. *Proteomics* **1**, 194–199
41. Nollau, P. and Mayer, B.J. (2001) Profiling the global tyrosine phosphorylation state by Src homology 2 domain binding. *Proc. Natl. Acad. Sci. USA* **98**, 13531–13536
42. Liu, Q. and Dreyfuss, G. (1995) *In vivo* and *in vitro* arginine methylation of RNA-binding proteins. *Mol. Cell. Biol.* **15**, 2800–2808
43. Kzhyshkowska, J., Schutt, H., Liss, M., Kremmer, E., Stauber, R., Wolf, H., and Dobner, T. (2001) Heterogeneous nuclear ribonucleoprotein E1B-AP5 is methylated in its Arg-Gly-Gly (RGG) box and interacts with human arginine methyltransferase HRMT1L1. *Biochem. J.* **358**, 305–314
44. Schurter, B.T., Koh, S.S., Chen, D., Bunick, G.J., Harp, J.M., Hanson, B.L., Henschen-Edman, A., Mackay, D.R., Stallcup, M.R., and Aswad, D.W. (2001) Methylation of histone H3 by coactivator-associated arginine methyltransferase 1. *Biochemistry* **40**, 5747–5756
45. Ma, H., Baumann, C.T., Li, H., Strahl, B.D., Rice, R., Jelinek, M.A., Aswad, D.W., Allis, C.D., Hager, G.L., and Stallcup, M.R. (2001) Hormone-dependent, CARM1-directed, arginine-specific methylation of histone H3 on a steroid-regulated promoter. *Curr. Biol.* **11**, 1981–1985
46. Strahl, B.D., Briggs, S.D., Brame, C.J., Caldwell, J.A., Koh, S.S., Ma, H., Cook, R.G., Shabanowitz, J., Hunt, D.F., Stallcup, M.R., and Allis, C.D. (2001) Methylation of histone H4 at arginine 3 occurs *in vivo* and is mediated by the nuclear receptor coactivator PRMT1. *Curr. Biol.* **11**, 996–1000
47. Wilkins, M.R., Gasteiger, E., Gooley, A., Herbert, B., Molloy, M.P., Binz, P.A., Ou, K., Sanchez, J.-C., Bairoch, A., Williams, K.L., and Hochstrasser, D.F. (1999) High-throughput mass spectrometric discovery of protein post-translational modifications. *J. Mol. Biol.* **289**, 645–657
48. Zhou, J., Mulshine, J.L., Unsworth, E.J., Scott, F.M., Avis, I.M., Vos, M.D., and Treston, A.M. (1996) Purification and characterization of a protein that permits early detection of lung cancer. Identification of heterogeneous nuclear ribonucleoprotein-A2/B1 as the antigen for monoclonal antibody 703D4. *J. Biol. Chem.* **271**, 10760–10766
49. Liu, C., Stewart, C.A., King, R.L., Danner, D.A., Dell'Orco, R.T., and McClung, J.K. (1994) Prohibitin expression during cellular senescence of human diploid fibroblasts. *Biochem. Biophys. Res. Com.* **201**, 409–414