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Protein *N*-Arginine Methylation in Subcellular Fractions of Lymphoblastoid Cells[1]

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Arginine methylation in RNA-binding proteins containing arginin- and glycine-rich RGG motifs is catalyzed by specific protein arginine *N*-methyltransferase in cells. We previously showed that lymphoblastoid cells grown in the presence of an indirect methyltransferase inhibitor, adenosine dialdehyde (AdOx), accumulated high level of hypomethylated protein substrates for the endogenous protein methyltransferases or recombinant yeast arginine methyltransferase [Li, C. *et al.* (1998) *Arch. Biochem. Biophys.* 351, 53–59]. In this study we fractionated the lymphoblastoid cells to locate the methyltransferases and the substrates in cells. Different sets of hypomethylated methyl-accepting polypeptides with wide range of molecular masses were present in cytosolic, ribosomal, and nucleus fractions. The methylated amino acid residues of the methyl-accepting proteins in these fractions were determined. In all three fractions, dimethylarginine was the most abundant methylated amino acid. The protein-arginine methyltransferase activities in the three fractions were analyzed using recombinant fibrillarin (a nucleolar RGG protein) as the methyl-accepting substrate. Fibrillarin methylation was strongest in the presence of the cytosolic fraction, followed by the ribosomal and then the nucleus fractions. The results demonstrated that protein-arginine methyltransferases as well as their methyl-accepting substrates were widely distributed in different subcellular fractions of lymphoblastoid cells.

Key words: adenosine dialdehyde, arginine methyltransferase, lymphoblastoid cell, protein methylation.

Many RNA-binding proteins involved in RNA processing and ribosome biogenesis including heterogeneous nuclear ribonucleoproteins (hnRNPs), fibrillarin, and nucleolin contain glycine- and arginine-rich motifs (GAR or RGG box). Frequently, the arginine residues in GAR or RGG motifs are modified as N^G-monomethylarginine (MMA) or N^G,N^G-dimethylarginine (asymmetric DMA) (*1–5*). The GAR domain appears to be essential for the RNA-binding activity of these proteins (*6*, *7*) and is involved in protein–protein interactions for hnRNPA1 (*8*) and nucleolin (*9*). It is still unclear why the arginine residues in the RGG box are specifically modified, and whether the arginine methylation in the RGG box may affect the function of the RNA-binding proteins. For instance, Rajpurohit *et al.* have shown that the binding activity of the methylated recombinant hnRNP-A1 was reduced as compared with its unmethylated form (*10*). On the other hand, methylation of a yeast hnRNP Hrp1p did not affect its specific RNA-binding activity (*11*). It was also demonstrated in yeast that the arginine methylation might be important for the nuclear export of hnRNP proteins (*12*). Therefore, it was proposed that this modification is involved in the recognition of protein-RNA complexes for nuclear export (*12*).

Systematic investigation of methyl-accepting polypeptides in cells can be achieved by growing cells in the presence of periodate-oxidized adenosine (adenosine dialdehyde, AdOx). AdOx is an inhibitor of *S*-adenosylhomocysteine (AdoHcy) hydrolase and can elevate the cellular level of AdoHcy, which inhibits the utilization by methyltransferases *S*-adenosylmethionine (AdoMet), the methyl group donor (*13*, *14*). In this way, methyl-accepting sites in polypeptides that would otherwise be irreversibly modified can be left unoccupied in cells, and analyzed later by *in vitro* methylation. We have used AdOx to treat human lymphoblastoid cells prepared from lymphocytes to accumulate high level of methyl-accepting polypeptides (*15*). Through *in vitro* methylation, many polypeptides in total extracts of AdOx-treated cells were methylated by the endogenous methyltransferases, while scarcely any polypeptides from

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Abbreviations: RGG box, arginine- and glycine-rich motif; AdOx, adenosine dialdehyde; RMT, arginine methyltransferase; PRMT, protein arginine methyltransferase; GST, glutathion-S-transferase; AdoMet, *S*-adenosylmethionine; hnRNP, heterogeneous nuclear ribonucleoproteins; DMA, dimethylarginine; MMA, monomethylarginine.

cells grown in the absence of AdOx were methylated. Moreover, a peptide R9 (GGRGRGGGF) derived from the RGG sequence of fibrillarin and the protein product of the *FMR-1* gene (FMRP; *16*) could specifically reduce the methylation in the protein substrates of the AdOx-treated cell extracts, indicating that the protein methyl-acceptors in lymphoblastoid cells might share similar arginine- and glycine-rich motifs (*15*).

The majority of the known GAR-containing substrates for type I protein methyltransferase are nuclear proteins (*e.g.*, some hnRNPs) or proteins that shuttle between the nucleus and cytoplasm (*e.g.*, hnRNPA1). However, the identities of the RGG-containing polypeptides and their cellular localization remain elusive. Moreover, the mammalian protein arginine methyltransferases that catalyze arginine methylation of proteins may be complicated, and there may be different enzymes in different cell types as well as subcellular compartments. Protein arginine *N*-methyltransferase activities have been detected biochemically or genetically in several systems. The methyltransferases responsible for the methylation of histone, recombinant hnRNPA1, and other nuclear proteins to form N^G,N^G-dimethylarginines were described in bovine brain, rat liver, and HeLa cells. They are large complexes with native molecular masses of 275–450 kDa (*5*, *17*, *18*). A rat protein-arginine *N*-methyltransferase gene (PRMT1) and the human homologue were cloned unexpectedly as genes encoding interacting proteins of the immediate-early gene product TIS21 (*19*) and the intracytoplasmic domain of interferon α, β receptors (*20*) respectively. It was thus postulated that PRMT1 with calculated molecular mass of 40 kDa might be the catalytic subunit of a large methyltransferase complex and be involved in certain signal transduction pathways (*19*, *21*). Moreover, two ubiquitously expressed mammalian homologues of PRMT1 with similar protein methyltransferase motifs, HRMT1L1 (PRMT2) (*22*, *23*), and PRMT3 (*24*), were identified. The arginine methyltransferase activities of the two gene products assayed *in vitro* were either low compared with PRMT1 (PRMT3, Ref. *24*) or could not be detected (PRMT2, Ref. *22*). Immunofluorescent analysis revealed that PRMT1 is located in the nucleus and PRMT3 in the cytoplasm, even though the interaction of PRMT1 and PRMT3 was revealed by yeast two-hybrid analyses (*24*). Recently, Chen *et al.* identified a p160-family co-activator (for nuclear hormone receptor)–associated arginine methyltransferase (CARM1) with sequence homology to PRMT1. The methyltransferase activity of CARM1 may be related to transcription regulation (*25*).

In order to better characterize the methyl-accepting polypeptides and the protein-arginine *N*-methyltransferases, in this study we fractionated the lymphoblastoid cells. We then analyzed the base-stable protein methyl-accepting substrates and the arginine methyltransferase activities in different subcellular fractions by *in vitro* methylation.

MATERIALS AND METHODS

Culture and Fractionation of Lymphoblastoid Cells—Lymphoblastoid cells were grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 37°C in a 5% CO_2 incubator. Cells were treated with 20 μM of AdOx for 72 h as described (*15*). The cells were harvested and fractionated by the procedures of Corbin *et al.* (*26*). Briefly, cells were washed with PBS, resuspended in buffer A (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 100 mM NaF, 10 ng/ml aprotinine, 1 mM PMSF, 1 mM dithiothreitol, 10 U/ml RNasin, and 0.5% NP-40), then further disrupted by passing through hypodermic needles (22 G) 10 times. The crude nuclear fraction was obtained by centrifugation at 1,000 ×*g* for 5 min. The supernatant was re-centrifuged at 100,000 ×*g* for 60 min. The pellet of the 100,000 ×*g* centrifugation was designated as the "ribosome" fraction and the supernatant as the cytosolic fraction.

Western Blotting—An equal amount of protein (30 μg) of each fraction was separated by 12.5% SDS-PAGE and subsequently transferred to nitrocellulose membranes (Gelman Science). The membranes were then blocked in 5% skimmed dry milk in TTBS (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.05% Tween 20) for 30 min, and incubated with primary Ab (1:1,000 dilution for anti-fibrillarin antisera or 1:5,000 dilution for anti-FMRP monoclonal antibody from Chemicon) for 1 h. They were then washed twice in TTBS and incubated with horse radish peroxidase-conjugated secondary antibody for 1 h. Chemiluminescence detection was performed using the Supersignal kit (Pierce) according to the manufacture's instructions. Lactate dehydrogenase activity was assayed according to a standard protocol (*27*)

Preparation of Recombinant Proteins—Recombinant GST-RMT1 was prepared from IPTG-induced, pGEX-RMT1 transformed *E. coli* (DH5α) cells as described by Gary *et al.* (*28*). Recombinant mouse fibrillarin with six-histidine tag was expressed in pET28-fibrillarin transformed JM109 (DE3) cells induced with IPTG at 25°C. Recombinant protein was purified under denaturing conditions through a Ni-NTA column (Qiagen) according to the procedures described by Pearson *et al.* (*29*).

In Vitro Protein Methylation—Different subcellular fractions (20–30 μg of proteins) were incubated with 4 μCi of [*methyl*-^{3}H]-AdoMet (60 Ci/mmol, Amersham) in the presence or absence of recombinant yeast GST-RMT1 arginine methyltransferase in a volume of 40 μl in Buffer B (25 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA). After 60 min of incubation at 30°C, the reaction was terminated by the addition of one-third of the volume of 3× SDS sample buffer, and the samples were subjected to SDS-PAGE (12.5% acrylamide) as described by Laemmli (*30*). The polyacrylamide gels were stained with Coomassie Brilliant Blue, destained, and treated with EN^{3}HANCE (Du Pont NEN). Subsequently, the gels were dried and exposed to X-ray film (Kodak, MS) at −75°C for 7 d if not indicated otherwise.

Acid Hydrolysis of Methylated Proteins and Analysis of Methylated Amino Acids—To analyze the methylated amino acids in the methyl-accepting proteins, the *in vitro* methylation reaction was quenched by the addition of equal volume of 25% of trichloroacetic acid. The proteins were precipitated by centrifugation, washed once with acetone, and dried. The polypeptides were hydrolyzed in 6 N HCl (Pierce) at 105°C in vacuum for 24 h. The hydrolysates were derivatized with phenylisothiocyanate (PITC, Pierce), then applied to a C18 column (Waters, PICO-TAC, 5 μm, 3.9 mm × 15 cm). The column was eluted at the flow rate of 1 ml/min by linear gradient starting with 100% eluent A (8.225 mM sodium acetate, 0.047% triethylamine, pH 6.4, 6% acetonitrile) to 13, 25, 33, 37, 39, and 40% eluent B

(60% acetonitrile in water) at 5, 10, 15, 20, 25, and 30 min, respectively. Fractions were collected every 0.25 min, and the radioactivities in samples of 100 μl were determined by liquid scintillation counting. Derivatized methyl amino acid standards (Sigma) were eluted under the same conditions.

RESULTS

Putative Arginine Methyltransferase Substrates in Three Subcellular Fractions of Lymphoblastoid Cells Not Treated by AdOx—We first prepared the nuclear, ribosomal and supernatant fractions from lymphoblastoid cells. The fractions were examined by Western blot analysis to validate the fractionation procedures. As expected, FMRP and fibrillarin were in the ribosomal (*27*) and nucleus (nucleolus, Ref. *2*) fractions respectively (Fig. 1). The activity of a cytosolic marker enzyme, lactate dehydrogenase, was also present predominantly in the cytosolic fraction (Fig. 1). We then performed *in vitro* methylation in the presence of [*methyl*-^{3}H]AdoMet. If the cells were grown in the absence of AdOx, virtually none of the polypeptides in all three subcellular fractions could be methylated by the endogenous methyltransferases *in vitro* (Fig. 2, lanes 1, 3, and 5). The results were consistent with the previous report on the methylation of the total extract of lymphoblastoid cells (*15*), except that a polypeptide of about 60 kDa in the ribosomal and cytosolic fractions was methylated (lanes 3 and 5). A predominant yeast protein arginine methyltransferase RMT1/HMT1 (*28*, *31*), can modify appropriate protein substrates to form MMA and asymmetric DMA and appeared to have broader substrate specificity than its mammalian homologue PRMT1, which might be regulated by a certain signal transduction pathway (*19*, *21*). We thus use recombinant yeast RMT1 to probe the presence of any potential arginine methyl-accepting sites undetected by the endogenous mammalian methyltransferases, since the *in vitro* reaction conditions might not be optimized for the more tightly regulated enzyme. Upon addition of exogenous yeast RMT1 enzyme, each of the three fractions showed a distinct pattern of methylation (lanes 2, 4, and 6). In the nuclear fraction (lane 2), 2 polypeptides with molecular mass of less than 19 kDa were heavily methylated, and about 12 other polypeptides with molecular mass of more than 32 kDa were detected. In the ribosomal fraction (lane 4), 4 polypeptides with molecular mass of 14, 20, 26, and 32 kDa were predominant. In addition, about 15 other polypeptides were also resolved in this fraction. Interestingly, the position of the 60 kDa polypeptide that could be methylated by the endogenous methyltransferase(s) could also be modified to a stronger extent by RMT1. In the cytosolic fraction (lane 6), the level of methylation was relatively low, but more than 20 polypeptides of different molecular mass were nevertheless detectable. Thus in all three subcellular fractions of lymphoblastoid cells, there were polypeptides that could be modified by the exogenous yeast RMT1 arginine methyltransferase. The methyl-accepting sites of these polypeptides probably could not be modified or were not fully methylated by the endogenous enzymes of lympho-

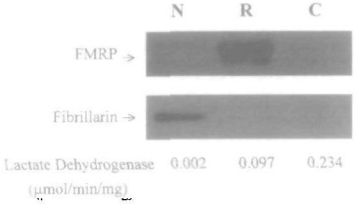

Fig. 1. **Analysis of subcellular fractionation of lymphoblastoid cells.** Lymphoblastoid cells were fractionated into cytosolic (C), ribosomal (R), and nucleus (N) fractions as described in "MATERIALS AND METHODS." An equal amount of protein (30 μg) of each fraction was separated by 12.5% SDS-PAGE and subsequently transferred to nitrocellulose membranes for Western blotting with anti-fibrillarin antisera or anti-FMRP antibodies as the first antibody and horseradish peroxidase–conjugated secondary antibodies followed by chemiluminescent detection. The lactate dehydrogenase activity in each subcellular fraction was indicated.

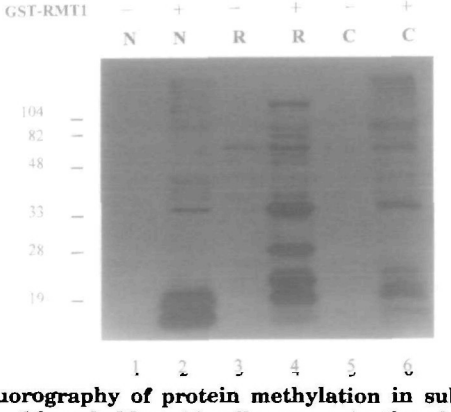

Fig. 2. **Fluorography of protein methylation in subcellular fractions of lymphoblastoid cells grown in the absence of AdOx.** Subcellular fractions (30 μg of proteins) of lymphoblastoid cells grown in the absence of AdOx were methylated with or without exogenous GST-RMT1 (6 μg) as described under "MATERIALS AND METHODS." The proteins were resolved by SDS gel electrophoresis and were fluorographed. Molecular markers (low range prestained protein marker from BioRad) are phosphorylase *b* (104,000), bovine serum albumin (82,000), ovalbumin (48,300), carbonic anhydrase (33,400), soybean trypsin inhibitor (28,300), and lysozyme (19,400).

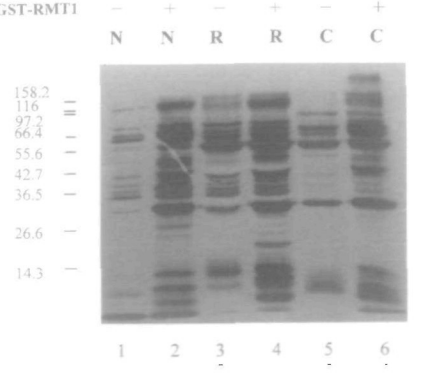

Fig. 3. **Fluorography of stable protein methylation in subcellular fractions of lymphoblastoid cells grown in the presence of AdOx.** Subcellular fractions (30 μg of proteins) of lymphoblastoid cells grown in the presence of AdOx were methylated with or without exogenous GST-RMT1 (6 μg). The proteins were resolved by SDS gel electrophoresis and were fluorographed. The figure shows the results of a seven-day exposure at −75°C. Positions of molecular markers (broad range protein marker from New England Biolab, including MBP-β-galactosidase [158,194], β-galactosidase [116,351], phosphorylase *b* [97,184], bovine serum albumin [66,409], glutamic dehydrogenase [55,561], maltose-binding protein [42,710], lactate dehydrogenase [36,487], triosephosphate isomerase [26,625], and lysozyme [14,313]) are marked on the left in thousands.

blastoid cells and thus were available for exogenous RMT1.

Putative Arginine Methyltransferase Substrates in Three Subcellular Fractions of Lymphoblastoid Cells Treated with AdOx—Cells were grown in the presence of the potent cellular methylation inhibitor adenosine dialdehyde (AdOx) to accumulate polypeptides with empty methyl-accepting sites, and the proteins were analyzed by *in vitro* methylation (*15*). Methyl-accepting polypeptides methylated by endogenous methyltransferases present in the same subcellular fraction appeared in all three fractions (Fig. 3; lanes 1, 3, and 5). The result indicated that hypomethylated protein methyl-acceptors and endogenous protein methyltransferases, whether protein-arginine *N*-methyltransferases or not, were present in all three fractions. In the nucleus fraction, fewer polypeptides appeared to be intensively methylated by the endogenous enzyme (lane 1) than in the other two fractions (lanes 3 and 5). The polypeptides methylated by the endogenous enzyme (lane 1) in the nucleus fraction could be methylated further by the recombinant yeast RMT1 (lane 2). For instance, some polypeptides with molecular mass of less than 32 kDa were barely detected by the endogenous enzyme but were heavily methylated by RMT1.

In the ribosomal fraction, the effect of exogenous RMT1 was less obvious than in the other two fractions. Comparison of the methylation patterns with (lane 4) and without (lane 3) exogenous RMT1 enzyme reveals only one extra methylated species, a polypeptide of about 20 kDa. Therefore, it is likely that the RMT1 and the endogenous enzymes in the ribosomal fraction recognized similar polypeptide substrates.

In the cytosolic fraction, certain polypeptides were methylated heavily (*e.g.*, 86, 65, 58, and 32 kDa) by the endogenous enzyme (lane 5) and were not further methylated by RMT1 (lane 6). However, RMT1 did methylate some extra polypeptides (*e.g.*, >200 kDa, 45–55 kDa). Moreover, some methylated polypeptides with identical mobilities were present in more than one subcellular fraction. For example, the 32 kDa polypeptides that can be heavily methylated by RMT1 appeared to be present in all three fractions prepared from cells grown in the absence of AdOx (Fig. 2). It could be the 32 kDa polypeptide reported previously in the total extracts of lymphoblastoid cells (*15*). On the other hand, a set of four polypeptides between 45–55 kDa were present in the nucleus and ribosomal fraction but not in the cytosolic fraction.

Analysis of the Methylated Amino Acids in Three Subcellular Fractions—To further characterize the base-stable methylated proteins in all three fractions, we analyzed the methylated amino acids in these subcellular fractions. AdOx-treated subcellular fractions were methylated with [*methyl*-^{3}H]AdoMet, hydrolyzed, and then the radioactively labeled methylated amino acids were analyzed by reverse-phase HPLC. The percentages of methylated arginines in total radioactivity are shown in Table I. In the cytosolic fraction, when proteins were methylated by the endogenous enzyme, 70.5% of the radioactivity recovered coeluted with dimethylarginine (DMA) and 14.9% coeluted with monomethylarginine (MMA). In the ribosomal fraction, 67.7% of the radioactivity recovered coeluted with DMA and 24.5% with MMA. In these fractions, about 90% of the radioactivities were incorporated into the arginyl residues. In the nucleus fraction, 41.0 and 20.0% of the radioactivity recovered coeluted with DMA and MMA. Interestingly, in the nucleus fraction, about 28.3% of the radioactivity eluted at a position corresponding to that of methyllysines, which was not detected in the other two fractions. In any event, under the experimental conditions, arginyl residues were the predominant methylation sites in the methyl-accepting polypeptides. Moreover, the ratios of dimethylarginine to monomethylarginine in each fraction were also rather constant (Table I).

Putative Arginine Methyltransferase Activity in Three Subcellular Fractions of Lymphoblastoid Cells—The above results revealed arginine methyltransferase activities in all three subcellular fractions of the lymphoblastoid cells. We thus used fibrillarin, an exogenous RGG protein substrate, to examine the presence of the arginine methyltransferase activity. Fibrillarin is a nucleolar protein containing 4.1 mol% of N^G,N^G-dimethylarginine (DMA), and the first 31 residues of this 34-kDa protein include 6 DMA (*2*). Since *E. coli* does not have the protein arginine modification system as in eukaryotes, the mouse fibrillarin protein expressed in *E. coli* could be an ideal methyl-accepting substrate for the protein-arginine *N*-methyltransferase in eukaryotic cells. We prepared lymphoblastoid subcellular fractions from cells without AdOx treatment and used them as the enzyme source. In these systems, the endogenous substrates for the endogenous enzyme should have been methylated to

TABLE I. **The formation of methylarginines in subcellular fractions of AdOx-treated lymphoblastoid cells.**

Subcellular fractions	Percentage of radioactivity recovered[a]		
	MMA	DMA	DMA/MMA
Cytosol	14.9 ± 2.1	70.5 ± 4.2	4.8 ± 0.9
Ribosome	24.5 ± 1.2	67.7 ± 1.1	2.8 ± 0.2
Nucleus	20.0 ± 1.3	41 0 ± 7.2	2.1 ± 0.5

[a]All values are the average ± standard deviation from four experiments.

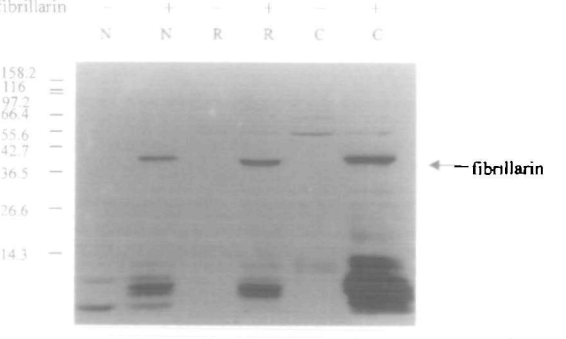

Fig. 4. **Protein-arginine *N*-methyltransferase activity in three subcellular fractions of lymphoblastoid cells using recombinant fibrillarin as a methyl-accepting substrate.** Subcellular fractions (30 μg of proteins) of lymphoblastoid cells grown in the absence of AdOx were used as the source of protein methyltransferase. Recombinant fibrillarin (5 μg) were included in each reaction as specific methyl-accepting substrates. Methylation reactions, protein electrophoresis and fluorography were performed as described under "MATERIALS AND METHODS." The location of fibrillarin on the gel is indicated by the arrow. The strong signals at the bottom of the gel might be due to methylation of degraded products of recombinant fibrillarin. Molecular weight standards were the same as those in Fig. 3.

the full extent (see Fig. 2) and should not interfere with fibrillarin methylation. The results clearly showed that all of the three fractions contained the methyltransferase activity catalyzing the transfer of methyl groups from AdoMet to fibrillarin (Fig. 4). The extent of fibrillarin methylation was the highest in the cytosolic fraction, followed by the ribosomal and the nucleus fraction. Similar experiments were performed with other cell lines such as neuroblastoma SK cells and hepatoma HepaG2 cells, and identical results were obtained (data not shown).

DISCUSSION

In this study, we analyzed methyl-accepting polypeptides and the protein-arginine *N*-methyltransferase activity in subcellular fractions of lymphoblastoid cells. The results are summarized in Table II, Which shows several interesting points. Firstly, methyl-accepting substrates for the endogenous enzymes in AdOx-treated lymphoblastoid cells were most abundant in the ribosomal fraction. Since addition of RMT1 did not increase the methylation level of most methyl-accepting polypeptides in the ribosomal fraction, it is likely that most of the substrates were methylated at the arginyl residues by the endogenous enzyme. Secondly, methyl amino acid analysis revealed that arginyl residues were the predominant sites for methylation in proteins. This conclusion is consistent with the result that recombinant fibrillarin protein was capable of competing off the methylation on various polypeptides in a concentration-dependent manner in all three fractions (data not shown). Thus the majority of the methylated polypeptides displayed by SDS-PAGE analysis were likely to be arginine-methylated. Thirdly, although the methylation level was relatively low in the nucleus fraction after the action of endogenous methyltransferase, there were plenty of substrates for yeast RMT1. One possible interpretation is that the nucleus fraction contained abundant hypomethylated substrates for arginine methyltransferase, yet the arginine methyltransferase activity was low. This assumption is consistent with the result that the arginine methyltransferase activity assayed by fibrillarin was lowest the nucleus fraction. Interestingly, the percentage of methylarginines in this fraction was also lower than that in the other two fractions.

As shown in Figs. 2 and 3, certain polypeptides with identical molecular masses appeared to be present in two or all three of the subcellular fractions. It is not clear whether the proteins of identical mass in different fractions were identical or not. Based on the results of immunoblotting in Fig. 1, which show that fibrillarin and FMRP were well separated, it is less likely that the overlapping of methyl-accepting polypeptides in different fractions was due to insufficient subcellular fractionation. Although *in vitro* analysis of the protein methylation in various subcellular fractions might not absolutely reflect the actual physiological situation, it is nevertheless an improvement over the reaction performed in total cell extracts (*15*, *32*). In that situation, the enzymes and the substrates from different cellular compartments might react, while this would not occur *in vivo*.

We have also analyzed the methylated amino acids on the methyl-accepting polypeptides after *in vitro* methylation. Methylarginines, including mono- and dimethylarginines, are the most abundant methylated amino acid species in all three fractions (Table I). Similar results were previously reported for PC12 cells, where 90% of known methylated amino acids are methylarginines (*32*). Interestingly, the ratio of dimethylarginine to monomethylarginine varied in all three fractions, being equal to 2.1 in the nucleus, 2.8 in the ribosome and 4.8 in the cytosolic fraction. On the other hand, the ratio of the dimethylarginine (71.5%) to monomethylarginine (18.3%) in the total extracts of PC12 cells would be about 3.9 (*32*). Different substrates are known to be methylated to different extents to mono- or dimethylarginines. For instance, the ratio of DMA to MMA in recombinant hnRNPA1 protein methylated by purified calf brain protein arginine methyltransferase increased with time and finally reached 2.4 (*33*). For mouse recombinant fibrillarin, the ratio of DMA to MMA was about 7 when methylated by GST-PRMT1 or GST-RMT1 (Lin and Li, unpublished data). However, only monomethylarginine could be detected in RGG-containing peptides when methylated either by purified or recombinant arginine methyltransferase (*16*, *28*, *34*). Therefore, one possible explanation for the differences in the ratios is that distinct substrates are present in various subcellular fractions and they could be methylated preferentially to mono- or dimethylarginines. However, it is also possible that the ratios of DMA to MMA resulted from different enzymes with distinct substrate specificity in these fractions.

We showed that the methyltransferase activity assayed by using fibrillarin as the methyl-accepting substrate was the strongest in the cytosolic fraction (Fig. 4). It remains to be determined whether, fibrillarin was methylated by the same or different arginine methyltransferases in different subcellular fractions of lymphoblastoid cells. Previous studies revealed specific cellular localization of several arginine methyltransferases. On the basis of immunofluorescent analysis, the PRMT1 arginine methyltransferase was located in the nucleus, while PRMT3 with sequence homology to PRMT1 was located in the cytoplasm (*24*). In any event, further analyses on the specific methyl-accepting substrates and characterization of arginine methyltransferase, including its regulation, cellular distribution and specificity, are crucial for the understanding of the predominant protein methylation widely present in eukaryotic proteins.

TABLE II. **Summary of the arginine methyl-accepting substrates and protein arginine methyltransferase activity in subcellular fractions of lymphoblastoid cells.**

Subcellular fractions	Methyl-accepting substrates[a] RMT			Methyltransferase activity[b] (fibrillarin)
	−		+	
Cytosolic	++	(85%)	+++	+++
Ribosomal	+++	(92%)	++++	++
Nucleus	+	(61%)	++++	+

[a]The relative intensities of the signals of methyl-accepting substrates in each subcellular fraction of AdOx-treated lymphoblastoid cells as shown in Fig. 3 were estimated by densitometer scanning. The number in parenthesis is the percentage of the methylarginines (mono- and dimethylarginine) as shown in Table I. [b]The extent of fibrillarin methylation was determined as in Fig. 4.

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