A Proteomic Analysis of Arginine-methylated Protein Complexes*

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Arginine methylation is a post-translational modification that results in the formation of asymmetrical and symmetrical dimethylated arginines (a- and sDMA). This modification is catalyzed by type I and II protein-arginine methyltransferases (PRMT), respectively. The two major enzymes PRMT1 (type I) and PRMT5 (type II) preferentially methylate arginines located in RG-rich clusters. Arginine methylation is a common modification, but the reagents for detecting this modification have been lacking. Thus, fewer than 20 proteins have been identified in the last 40 years as containing dimethylated arginines. We have generated previously four arginine methyl-specific antibodies; ASYM24 and ASYM25 are specific for aDMA, whereas SYM10 and SYM11 recognize sDMA. All of these antibodies were generated by using peptides with aDMA or sDMA in the context of different RG-rich sequences. HeLa cell extracts were used to purify the protein complexes recognized by each of the four antibodies, and the proteins were identified by microcapillary reverse-phase liquid chromatography coupled on line with electrospray ionization tandem mass spectrometry. The analysis of two tandem mass spectra for each methyl-specific antibody resulted in the identification of over 200 new proteins that are putatively arginine-methylated. The major protein complexes that were purified include components required for pre-mRNA splicing, polyadenylation, transcription, signal transduction, and cytoskeleton and DNA repair. These findings provide a basis for the identification of the role of arginine methylation in many cellular processes. ***Molecular & Cellular Proteomics 2:1319–1330, 2003.***

Protein arginine methylation is a post-translational modification that adds monomethyl or dimethyl groups to the guanidino nitrogen atoms of arginine (1). The enzymes responsible for protein arginine methylation have been classified in two major classes; type I enzymes promote the formation of asymmetrical ω-N^G,N^G-dimethylated arginines (aDMA),[1] and type II enzymes catalyze the formation of symmetrical ω-N^G,N'^G-dimethylated arginines (sDMA) (1). ω-N^G-Monomethylarginine is thought to be an intermediate formed by both enzyme types. The metabolic cost of methylation is high, requiring the use of 12 ATP molecules/methylation event (1). The fact that evolution has retained such an "expensive" reaction underscores the biological importance of this post-translational modification (2). There are now at least five type I protein-arginine methyltransferases in mammals; PRMT1 (3), PRMT2 (4), PRMT3 (5), CARM1 (PRMT4) (6), and PRMT6 (7); and one type II, PRMT5 (8). Recently, a new arginine methyltransferase was identified and by homology is likely a type I PRMT (9).

Myelin basic proteins (MBPs) and histones are among the first proteins shown to contain dimethylated arginines (10, 11). MBP has been shown to contain sDMA, but the enzyme or the function of this post-translational modification remains unknown. Histones have been shown to be methylated by PRMT1 and CARM1 *in vivo* (12–16). Histone-arginine methylation is thought to contribute to the histone code (17). Another class of dimethylated proteins includes RNA-binding proteins (18, 19). Arginine-glycine-rich sequences have been suggested and have been shown to contribute to the RNA binding activity (20–22). Thus, the methylation of these arginines would be predicted to alter or regulate their RNA binding activity, but the evidence for this has been lacking. It has been shown that arginine methylation regulates protein localization (2). It was first shown by Shen and co-workers (23) that the removal of the yeast methyltransferase Hmt1p causes the nuclear retention of two hnRNPs, Npl3p and Hrp1p. Subsequently it was shown that arginine methylation regulates the import of Npl3p (24) and the protein localization of hnRNP A2 (25), Sam68 (26), and p80-coilin (27, 28).

Arginine methylation has been shown to regulate protein-

[1] The abbreviations used are: aDMA, asymmetrical ω-N^G,N^G-dimethylated arginine(s); sDMA, ω-N^G,N'^G-dimethylated arginine(s); DMA, dimethylated arginine; PRMT, protein-arginine methyltransferase; MBP, myelin basic protein; hnRNP, heterogeneous nuclear ribonucleoprotein; snRNP, small nuclear ribonucleoprotein; SMN, survival motor neuron; EWS, Ewing sarcoma protein; ATM, ataxia telangiectasia mutated protein; CPSF, cleavage and polyadenylation specificity factor; TLS, translocated liposarcoma protein; LC/MS/MS, liquid chromatography-tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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protein interactions (29). The discovery that Sm proteins are methylated (30) and the discovery that the product of the spinal muscular atrophy gene product SMN associates with methylated Sm proteins (31) led to the proposal that arginine methylation may be a signal that targets proteins. In the case of small nuclear ribonucleoprotein (snRNP) particle assembly, arginine methylation by the PRMT5 methylosome (32, 33) has been proposed to be the signal for the recognition and targeting to the SMN protein complexes (31).

Although the role of arginine methylation in signal transduction was proposed in 1998 (34), few signaling proteins have been identified to be arginine-methylated. The inhibition of PRMT1 by antisense quenches the interferon signaling (35), and similarly arginine methylation of STAT1 has been shown to be required for interferon signaling (36). Moreover, the methylation of Sam68 peptides prevents their association with SH3 domains (29).

In the present manuscript, we utilized four arginine dimethyl-specific antibodies to purify arginine-methylated protein complexes. The proteins were identified by LC/MS/MS, and the protein sequences were searched for neighboring RG sequences that match the epitopes used to generate the peptide antibodies. We report the identification of over 200 proteins that are putatively arginine-methylated. These include RNA-binding proteins, transcription and polyadenylation factors, cytoskeleton proteins, as well as proteins involved in signal transduction and DNA repair. These data will help elucidate the role of arginine methylation in many cellular processes.

EXPERIMENTAL PROCEDURES

Antibodies—SYM10 and ASYM24 have been described previously (26, 27) and are distributed by UBI Inc. (Lake Placid, NY). SYM11 and ASYM25 were generated by immunizing rabbits with the peptides KAAILKAQVAAR^{sDMA}GR^{sDMA}GR^{sDMA}GMGR^{sDMA}G and KFGGR^{aDMA}GGGR^{aDMA}GGGR^{aDMA}GGFGGR^{aDMA}GGR^{aDMA}G, respectively. Polyclonal antibodies were generated by using New Zealand white rabbits injected with peptides coupled to keyhole limpet hemocyanin (Sigma).

Mass Spectrometry—HeLa-S3 (5×10^8) cells were obtained from Biovest International Inc./National Cell Culture Center (Minneapolis, MN) and lysed in a buffer containing 1% Triton X-100 (Roche Applied Science), 20 mM Tris, pH 7.4, 150 mM NaCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.01% phenylmethanesulfonyl fluoride. Endogenous methylated proteins were immunopurified from the cell lysate using 1 mg of the respective polyclonal methyl-specific antibody coupled to 1 g of protein A-Sepharose (Sigma). After extensive washings with lysis buffer and phosphate-buffered saline, pH 7.4, the bound proteins were eluted with 500 μl of 1× phosphate-buffered saline containing 250 μM corresponding immunogenic peptide. Eluted proteins were dialyzed against water overnight, lyophilized, and identified using trypsin digestion and LC/MS/MS sequencing of peptides. Specifically, proteins were directly digested (not reduced or alkylated) with 20:1 protein:trypsin (mass ratio) for 16 h at 37 °C. LC/MS/MS experiments were conducted on a Qstar Pulsar *i* instrument configured with a Protana nanospray source to which an Ultimate LC system was coupled (LC Packings/Dionex). A 75-μm inner diameter by 15 cm reverse-phase C_{18} PepMap™ column (LC Packings) operated at a flow rate of 200 nl/min with a gradient of 5–15% B (0–5 min), 15–50% B, 5–50% B (5–40 min), and 50–80% B (40–50 min). (Solvent A is 0.05% aqueous formic acid, and Solvent B is 0.05% formic acid in acetonitrile.) All solvents were high pressure liquid chromatography grade (Fisher). An information-dependent acquisition experiment was conducted using a 1-s survey scan and two 2-s pendant MS/MS scans incorporating mass-dependent ion bunching for sensitivity enhancement (pulsar mode). Some of the eluted proteins were resolved by SDS-PAGE and revealed by Coomassie Blue R-250 staining. Bands corresponding to appropriate molecular weights were excised, in-gel digested with trypsin, and analyzed on a Voyager DE-STR reflectron MALDI-TOF using a-cyano-4-hydroxycinnamic acid as the matrix in a standard dried droplet sample preparation protocol.

RESULTS

To identify dimethylated arginine-containing cellular proteins, immunoprecipitations were performed with dimethyl-specific antibodies. SYM10 is an antibody that we generated previously against peptide R^{sDMA}GR^{sDMA}GR^{sDMA}GR^{sDMA}G. Using enzyme-linked immunosorbent assay, we have shown that SYM10 could effectively distinguish sDMA *versus* aDMA, even at low dilutions of 1:500 (27). Further analysis by enzyme-linked immunosorbent assay helped us define the SYM10 epitope as at least two, preferentially non-contiguous, sDMA-Gs in a given peptide, which is consistent with the fact that SYM10 does not recognize a peptide derived from MBP that harbors a single sDMA-G (27). The epitope for SYM10 diminishes in PRMT5 small interfering RNA-treated cells demonstrating that PRMT5 is an enzyme that contributes to the SYM10 epitope (27). We have shown previously that one of the complexes immunoprecipitated by SYM10 was the spliceosomal snRNP core proteins, which include known sDMA-containing proteins SmB/B′, D1, and D3 (27). Among the additional proteins present in the SYM10 immunoprecipitate, we identified p80-coilin as being an sDMA-containing protein (27). A second antibody, SYM11, was generated against KAAILKAQVAAR^{sDMA}GR^{sDMA}GR^{sDMA}GMGR^{sDMA}G and also specifically recognizes sDMA-containing peptides (data not shown), as determined by the methodology described above for SYM10. SYM10 and SYM11 each recognize specific protein patterns as determined by immunoblotting (Fig. 1). The recognition patterns are distinct but partially overlap as the Sm proteins are both recognized by these antibodies (Fig. 1). ASYM24 was generated by using the peptide KGR^{aDMA}GR^{aDMA}GR^{aDMA}GR^{aDMA}GPPPPPR^{aDMA}GR^{aDMA}GR^{aDMA}GR^{aDMA}G as an antigen as reported previously (26). ASYM24 recognizes aDMA specifically and was shown to recognize Sam68, an RNA-binding protein (26). The epitope for ASYM24 diminishes significantly in PRMT1$-/-$ cells, demonstrating that the major enzyme that contributes to the ASYM24 epitope is PRMT1 (26). ASYM25 was generated by immunizing rabbits with the peptide KFGGR^{aDMA}GGGR^{aDMA}GGGR^{aDMA}GGFGGR^{aDMA}GGR^{aDMA}G. ASYM25 is aDMA-specific and has a different specificity than ASYM24 as demonstrated by immunoblotting (Fig. 1).

At least two large scale immunopurifications were performed with HeLa-S3 cell extracts using each of the dimethyl-

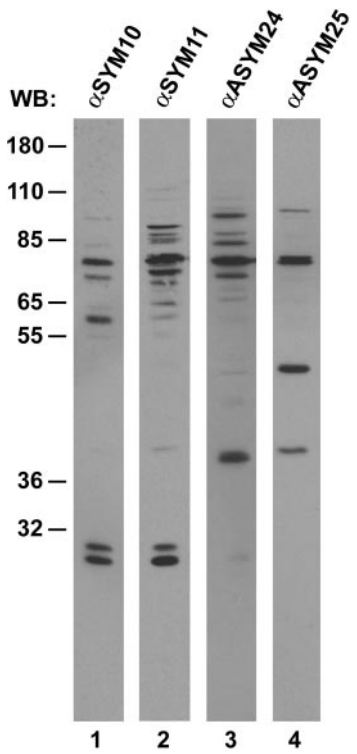

FIG. 1. **Immunoblotting profiles of methylarginine-specific antibodies used in this study.** HeLa cell lysates were immunoblotted with the indicated methylarginine-specific antibodies. The migration of the molecular mass markers is shown on the *left*. The migration of SmB/B′ and Sm D1–D3 is shown. *WB*, Western blot.

arginine-specific antibodies. One control immunoprecipitation was performed using the protein A-Sepharose resin alone (data not shown). The bound protein complexes were eluted with the respective immunogenic peptides. The eluted proteins were digested with trypsin, and the peptides were separated and identified by LC/MS/MS, which is a mass spectrometry technique particularly suitable to the analysis of complex protein mixtures. In the case of SYM10, predominant bands visualized by SDS-PAGE were also identified by using MALDI-TOF mass spectrometry (as indicated by a + sign in Table I). The resulting peptide mass maps were internally calibrated using three molecular weight standards and were submitted to Mascot (Matrix Science Ltd., London) search algorithms (with a 30-ppm mass tolerance with acrylamide adduction chemistry for cysteines) to query the National Center for Biotechnology Information non-redundant (NCBI-nr) database. These results were then confirmed using the Protein Prospector tools at University of California-San Francisco (prospector.ucsf.edu), and the identifications given a MOWSE score greater than 1000 were considered positive. All proteins that were scored positive using this procedure were also identified using LC/MS/MS, validating this approach. Hence, only LC/MS/MS was used for the remainder of the analysis. LC/MS/MS data were searched using Mascot against the National Center for Biotechnology Information non-redundant (NCBI-nr) database (release date of April 5, 2003) with a peptide mass tolerance of 100 ppm and a fragment mass tolerance of 0.1 Da as well as allowing for two missed cleavages. A protein was scored positive by LC/MS/MS if it was not found in the experiment performed with protein A-Sepharose alone and if 1) it was identified in more than one analysis, 2) the number of unique peptides was greater than three, or 3) the amino acid sequence contained two RG repeats separated by less than 10 amino acids. The identified proteins were grouped according to their known or putative functions (Tables I–IV). Proteins that were previously reported to be methylated are marked with ● in Tables I–IV. The proteins that contain the RG repeats represent the epitopes for our DMA-specific antibodies and are likely to contains sDMA or aDMA (shown in bold characters in Tables I–IV). The proteins that are devoid of RG motifs are likely co-purifying proteins.

Pre-mRNA Processing

SYM10 and SYM11—A fraction of the purified proteins with SYM10 and SYM11 includes proteins associated directly or indirectly with pre-mRNA splicing (Tables I and II). The Sm B, B′, D1/D3, and U6 small nuclear RNA-associated Sm-like proteins 4 and 8 contain RG repeats that have been shown to contain sDMA (30, 37) and are known epitopes for SYM10 (27) and predicted epitopes for SYM11. The entire spectrum of core Sm proteins (U1A, U2A′, SmB/B′, U2B′, SmD1–3, and SmE/F/G) and several snRNP-specific factors were affinity-purified, indicating that all assembled snRNPs are likely purified with SYM10 (Table I). Several snRNP co-purifying proteins were obtained including U1–70K, SAP155, U5–102K, U5 snRNP-specific protein, U5 snRNP 200-kDa helicase, and Prp8 (38). The serine-arginine (SR) kinase hPrp4 and SR proteins ASF/SF2, 9G8, SRm300, and SRp20 were likely co-purified with the snRNPs (39). However, 9G8, SRm300, and U1–70K contain RG repeats that are candidate epitopes for arginine methylation and SYM10 and SYM11 (Tables I and II).

The SYM10 and SYM11 antibodies purified the splicing factor "KH-type splicing regulatory protein" (KSRP), a homolog of zipcode-binding proteins ZBP1 and ZBP2 (40–42). The presence of RG repeats suggests that they contain sDMAs. The gene for *TLS* (Translocated in LipoSarcoma) or *FUS* is rearranged in human myxoid liposarcoma (43). The TLS protein has been shown to function as a splicing factor and is also implicated in DNA repair (44, 45). TLS was shown to be a substrate of PRMT1 (46) and shown to contain aDMA (47). Thus, TLS most likely represents a co-purifying protein for SYM11. Another gene product often associated with chromosomal translocation was identified, namely, the Ewing sarcoma protein EWS (48). The EWS protein has been shown to contain dimethylated arginines; however, it has not been shown whether the modification was symmetric or asymmetric (49). Our data suggest that EWS contains sDMA because of its presence in SYM10 immunoprecipitations (Table I). P80-coilin is the marker for Cajal bodies, and this nuclear structure

TABLE I
Proteins identified with the SYM10 sDMA-specific antibody

Function	Accession	Protein	SYM10 epitope	a.a	MALDI-TOF
Pre-mRNA Processing	4759156	U1 A	-	282	+
	4507121	U2 A'	-	255	+
	134037	**•Sm B/B'**	PGRGGPPPPMGRGAP	240	+
	4507123	U2 B"	-	225	
	5902102	**•Sm D1**	AGRGRGRGRGRGRGRGRGG	119	+
	4759158	Sm D2	-	118	
	4759160	**•Sm D3**	ARGRGRGMGRGN	126	+
	312005	Sm E	-	81	
	4507131	Sm F	-	86	
	4507133	Sm G	-	76	
	6912486	**•U6 snRNA-associated Sm-like protein 4, LSm4**	KGRGMGGAGRGVFGGRGRGIPGTGRGQ	139	
	7706425	U6 snRNA-associated Sm-like protein, LSm8	-	96	
	4507119	**U1-70K**	HKRGERGSERGRDEARG	614	+
	11360330	U4/U6-associated RNA splicing factor	-	682	
	2708305	hPrp4	-	522	
	2463577	Prp8	-	2335	
	24212088	U5 snRNP-associated 102 kD protein, U5-102K	-	941	
	4759280	U5 snRNP-specific protein, 116 kD	-	972	
	12643640	U5 snRNP 200 kD Helicase	-	1701	+
	338043	**Splicing Factor ASF/SF2**	GRGTGRGGGGGGGGAPRGR	278	
	3929380	**Splicing Factor 9G8**	RGRRYSRSRSRSRGRR	238	
	4506901	Splicing Factor SRp20	-	164	
	6912654	Splicing Factor 3b, Subunit 1 (SAP155)		1304	
	4504865	**KH-type splicing regulatory protein, KSRP / ZBP2**	GRGRGRGQG	711	
	585632	**•p80-Coilin**	GRGMRGRGRGRG	576	+
	544261	**•RNA-binding protein EWS**	RGGRGGGRGG	656	+
	448295	**•TLS protein**	FNRGGGNGRGGRGRGGP	260	+
Protein Translation	5901926	CPSF5, 25 kD subunit	-	227	
	5901928	**CPSF6, 68 kD subunit**	PQGGRGRGRFPGA	551	+
	18203334	CPSF, 100 kDa subunit	-	579	
	3183544	Polyadenylate-binding protein 1 (PABP1)	-	636	
	4505575	Polyadenylate-binding protein 2 (PABP 2)	-	633	+
	346208	eIF-4 gamma		1396	
DNA Transcription	5901962	MYST histone acetyltransferase 2	-	611	
	107932	**Transcription Factor TFEB**	RGGRGSGRGADGGREGR	514	
	4827071	**Zinc finger protein 9**	GGGRGRGMRSRGRGG	177	
Receptors and Signalling	219406	**Alpha2CII-adrenergic receptor**	RRGALRRGGRR	458	
	9506745	**Urotensin II Receptor**	LRGRVRGPGSGGGRGP	389	
	4502371	Breast cancer antiestrogen resistance 3, RasGEF	-	825	
	6740102	Crk-associated substrate p130Cas	-	870	
	28373065	**Potassium voltage-gated channel**	SGRGRVLLNSAAARGD	932	
Cytoskeletal Proteins	4502961	**Alpha 1 type VII collagen precursor**	PRGERGEPGIRGE	2944	
	22652113	**Alpha 1 type XXII collagen**	ERGEKGTRGEKGERGL	1626	
	5902122	**Spectrin, beta, non-erythrocytic 2**	PRGERQTRTRGP	2390	
	1346343	**Keratin, type II cytoskeletal 1**	ARGGGRGSGF	644	
	4508019	**Bassoon**	ARGPHGGPSQPTGPRGL	3926	
Apoptosis	7019477	HtrA2	-	458	
	2134780	Apoptosis Inhibitor IAP	-	497	
	292059	Mortalin-2 (Mitochondrial Heat Shock Protein 70 kD 9B)	-	679	
Enzymes	13623199	ATP citrate lyase	-	1101	
	21361331	Carbamoyl-phosphate synthetase 1	-	1500	
	23503239	**NAD-dependent aldehyde dehydrogenase**	GRGLDGAVDMGARGA	802	
Others	2342526	**IgE autoantigen**	ERGGERGSGRRGA	757	
	6912454	**Extra Spindle Poles like 1**	RRGTASRGRGRA	1795	
	21740275	**Hypothetical Protein**	ARGAGRGSARAARRARRG	459	
	7243183	**KIAA1401**	GGRHRGRGSAQRDGKGR	853	+
	20520991	**KIAA0294**	RGTRGTRGTRGTAGNG	1405	
	3882183	**KIAA0731**	FRGRGRGRGRGRGRGRGGT	1096	

is associated with snRNP assembly and thus splicing (50). We and others have shown that the RG repeats of p80-coilin are symmetrically dimethylated on arginines (27, 28).

ASYM24 and ASYM25—Three RNA helicases containing RG repeats were purified with ASYM25 (Table IV). One of these helicases, RH70, co-purifies with the U1 snRNP (51). In addition, several RNA-binding proteins were identified including Sam68, the Src substrate in mitosis, which has been shown to function in various aspects of RNA metabolism (26). HnRNP Q2 protein was purified with ASYM24 (Table III) suggesting that it contains aDMA. The RG sequences of hnRNP Q interact with SMN and may link SMN to the spliceosome (52).

TABLE II
Proteins identified with the SYM11 sDMA-specific antibody

Function	Accession	Protein	a.a.	SYM11 epitope
Pre-mRNA Processing	36495	**•SmB/B'**	218	PGRGGPPPMGRGAP
	5902102	**•Sm D1**	119	KREAVAGRGRGRGRGRGRGRGRGGPRR
	4759160	**•Sm D3**	126	QGSGAGRGKAAILKAQVAARGRGRGMGRGN
	88959	**U1- 70k**	614	HKRGERGSERGRDEARG
	1710627	**HnRNP A3**	379	GSQRGRGGGSGNCMGHRGNF
	27481414	**Similar to hypothetical RNA- binding protein KIAA0117**	425	RGSPPEDYRLGQVASSLFRGEHHSRGGTGR
	4504865	**KH-type splicing regulatory protein KSRP**	711	GMPPGGRGRGRGQGNW
	19923466	**Splicing coactivator subunit SRm 300**	2752	RGRSHSRSPATRGRSRSRTPARRGR
Protein Translation	25453474	**Eukaryotic translation elongation factor 1 delta isoform 1**	647	ARRGRRDRRGRN
DNA & Transcription	22094135	**Histone methyltransferase DOT1L**	1537	ARGDCVPSHGQDSRRRGRRK
	4505117	**Methyl-CpG binding domain protein 2 isoform 1**	411	GRGGGVCGRGRGRGRGRGRGRGRGRPP
	22653669	**Transcription terminator interacting protein 5**	1878	ITGKRGRPRNTEKAKTKEVPKVKRGRGRPP
	2723380	**DSIF p160 / transcription elongation factor**	1087	PSAGGQRGGFGSPGGGSGGMSRGRGR
	16554587	**TFIIF-associating CTD phosphatase I**	961	SAAGGRGPRGHKR
	13540590	**C/EBP induced protein**	453	MAGAAAGGRGGGAWGPGRGGAGGLRRGCSP
	4758600	**Interleukin enhancer binding factor 1 (ILF1)**	655	GTTRARGRGRGAGGGSRRALGRGPPGGPRV
	24234747	**Interleukin enhancer binding factor 2 (ILF2)**	390	MRGDRGRGRGGRFGSRGGPG
	256299	p98=Rel / NF-kappa B p105 homolog	900	-
Receptors and Signaling	4506569	**Roundabout 1 isoform a; ROBO1**	1651	EEEDERGPTPPVRGAASSP
	1351829	**Alpha-2A adrenergic receptor**	450	RGPRGKGKARASQVKPGDSLPRRG
	30353923	**Prostate derived STE20- like Kinase PSK**	1462	RGGFPGPPLRKGESRGRGGK
	4504795	**Inositol 1,4,5-triphosphate receptor, type 3**	2671	VGNRGTFIRGYKAMV
	4507157	**Mosaic receptor SorLA / LRII**	2214	WARGDARGAS
	1497931	Ataxia- telangiectasia / ATM	3056	-
Cell Cycle	940536	**P1 Cdc21 protein**	923	RGSRRGRATPAQTPRSEDARSSPSQRRRGE
Cytoskeletal Proteins	1346343	**Keratin, type II cytoskeletal 1**	644	ARGGGRGSGFGRG
	22652113	**Alpha 1 type XXII collagen**	1626	ERGEKGTRGEKGERGL
	18375518	**Alpha 1 type XI collagen isoform A preproprotein**	1806	ARGVAGKPGPRGQRGPTGPRGSRGARGPT
	4502961	**Alpha 1 type VII collagen precursor**	2944	PRGERGEPGIRGEDGRPGQEGPRGLTGPPGSRGERG
	435476	**Cytokeratin 9**	623	GRGSRGG
	4803663	**Ankyrin B**	3925	RGNTNMVKLLLDRGG
	5902122	**Spectrin, beta, non-erythrocytic 2**	2390	PRGERQTRTRGP
	4557703	**Keratin 2a**	645	MSCQISCKSRGRGGGGGGFRGFSSGSAVVS
	13435369	**Desmocollin 3 isoform Dsc3b preproprotein**	839	NQTLESCRGAGHHHTLDSCRGGHTE
	19115954	Dynein, axonemal, heavy polypeptide 5	4624	-
	13376204	E- catherin binding protein E7	491	-
Enzymes	3915598	**DNA-3-methyladenine glycosylase**	298	EDEAAHSRGGRQTPRNRGMF
	15076827	**Pentachlorophenol hydroxylase (Pcph)**	428	VRGKLHQPEEVQRGSFYA
	118090	Peptidl-prolyl cis-trans isomerase B precursor	208	-
	224517	Kinase, phosphoglyserate	417	-
	26665879	**CTAGE-2 / chromosome segration ATPase**	754	RGGGRGSRGPGNPPDHQITKERG
	3121981	Probable ATP- dependent RNA helicase DDX10	875	-
Others	6841194	**HSPC272**	2011	TSRLSGNRGV QYTRLAVQRG
	7717446	**PRED59**	211	AAGGQTRGRRWDRGGPGGPC
	292059	MTHSP75	679	-
	11415038	**Solute carrier family 22 member 3/organic cation transporter 2**	556	WNRTAPASRGPEPPERRGRC
	5042405	**BC282485_1**	477	LYKRGGRGSRGRGRPARPSP
	4503469	Early endosome antigen 1, 162kD	1410	-
	21553341	Synaptic nuclei expressed gene 2 nesprin 2	6885	-
	11877243	SSF1 / P2YII chimeric protein	794	-
	338490	52-kD SS-A / Ro autoantigen	475	-
	7513172	**N-chimerin homolog F25965_3**	903	YRAAPPAYGRGGELHRGSLY
	20539645	**Similar to mKIAA0038 protein**	228	AYSSFGSGRGSRGSAGGHGS
	14572619	Novel, protein, KIAA1769	2567	-
	6760015	**Brain protein**	344	PADDGAGPGGRGPRGRARGD
	3882199	**KIAA0739 protein**	1130	RTHGQKHRRRGRGKGASQGE
	10047331	**KIAA1627 protein**	468	RGGGRGGTSAGRGRERNRSNFRGERGGFRGGRGG
	3882183	**KIAA0731 protein**	1096	GGARASFRGRGRGRGRGRGRGGTRTHFD
	14017861	**KIAA1822 protein**	533	RGRPTAAPPAPTPRPARPTQQPGSRRGGGRRRG
	4204880	Heat shock protein	639	-
	11321605	**Smcx homolog, X chromosome, XE169**	1560	GSRARGRALERRRRRKVDRGGEGD
	29123596	SET binding factor 2	1849	-
	7512605	**Hypothetical protein DKFZp434H244.1**	845	RFELRRGSGGRGRFRGGDGV
	22044805	**Hypothetical protein XP_065829**	505	CLETAVLRGRRARGRFRGST
	18593376	**Hypothetical protein XP_097805**	161	AAASGARRHRGRGSIGGPFH
	27480502	**Hypothetical protein XP_209301**	261	PARGQHRTQPLRGRGWRGPL
	27486452	**Hypothetical protein XP_211272**	170	SGRTVRGSVSWKRPEQAAPETGRGPAVARG
	22044805	**Hypothetical protein XP_065829**	505	CLETAVLRGRRARGRFRGST
	27486452	**Hypothetical protein XP_211272**	170	TGRGPAVARG
	27479241	**Hypothetical protein XP_212123**	103	SERGQPTRGR
	18601673	**Hypothetical protein XP_047499**	232	SGARGGGRGARARRPGQRGR
	24432052	**Hypothetical protein FLJ25348**	696	GRGSIAPRGISAWQRPPRGRGR
	21389373	Hypothetical protein FLJ25359	506	-
	30156226	**Similar to hypothetical protein FLJ35782**	763	LRVARGLLATRVAGIRNRGNWRDG
	19913516	**Unknown (protein for MGC:26598)**	406	KRWGRRGRGL
	22477301	**Unknown (protein for MGC:46140)**	514	IEECRILRGPDGLSRGCAFV
	10435310	**Unnamed protein product**	193	TSVRDRRGDR HRGEGHVETE
	14336692	**Unknown**	321	PGGGAARGGP HLGGCGSAAR SGRGAGRGGA

TABLE III
Proteins identified with the ASYM24 aDMA-specific antibody

Function	Accession	Protein	a.a	ASYM24 epitope
RNA Processing	20450941	**RR M containing RNA-binding protein**	593	RGRGAAGNR
	15809588	**HnRNP Q2**	588	RGGPGSARGV RGARGGAQQQ RGRGVRGARG
	29733020	**RRM similar to REKIN cDNA**	425	GQVASSLFRGEHHSRGGTGR
DNA and Transcription	181488	**Zinc finger DNA-binding protein**	1902	YVYVRGRGRG
	12697318	**PBX4 protein**	330	PEKRGRGG
	1388162	**Interleukin enhancer binding factor 2 (ILF 2)**	609	TRARGRGR
Protein Translation	484950	**Valine-tRNA ligase**	1051	ALKERGLFRG
	20380061	**Likely ortholog of mouse variant polyadenylation protein CSTF-64**	616	RGMETCAMETRGMEARGMDARGLEMRG
	226021	**Nuclear protein with sequence homology to translation initiation factor eIF-4A**	594	RSRGRGGMK
	15029520	histidyl-tRNA synthetase	506	-
Receptor and Signaling	7446458	**Protein kinase (EC 2.7.1.37) N beta**	889	RGRGELASE
	4507009	**Solute carrier family 25, member 14 isoform UCP5L**	325	EGTRGLWRGV
	13632400	**Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing beta**	1634	TGRGRGMV
	631146	**FLT3/FLK2 ligand (clone S109**	245	RGESPARGCI AWTQRKLARG
	7768779	**Transient receptor potential-related channel 7, novel putative Ca2+ channel**	1503	GLRGRGSL
Cytoskeletal	126363	**Alpha-1 chain precursor (Laminin A chain)**	3075	HRGKLPAGSD RGPRPLVAPC
	4502955	**Alpha 5 type IV collagen isoform 1**	1685	GLPGDRGPPG PPGIRGPPGP
	2119157	**Collagen alpha 1(XIX) chain precursor**	1142	PPGKEGQRGR RGKTGPPGKP
	18765746	**Alpha 1 type XVIII collagen isoform 3 precursor; endostatin**	1473	RGPRGFPGPP GGMRGIRGAD
	8953371	**BA448E12.1 (collagen, type IV, alpha 6)**	1116	GLKGARGDRG
	190394	**Profilaggrin**	1084	IRGHPGSRRG
	13518037	**Matrilin 2 precursor**	956	AEARERSRGR SISRGRHART
	27764561	**TPA: keratin 1b**	502	RGGGARGRSG
	27720593	**Similar to cartilage intermediate layer protein**	1069	QRASRGGLRR RGSMAPLRFS
	32140760	**Collagen XXVII proalpha 1**	1860	PPGPPGDRGP VGDRGDRGEP
	338440	**Spectrin Rouen (beta-220-218) mutant coding sequence**	2106	PTTASRGGRR DSRGGSSFPP
	135448	tubulin beta-1 chain	444	-
	4505037	**Latent transforming growth factor beta binding protein 4 / LT BP-4**	1587	RGPGGRGLLR
Enzyme	4503021	**Carnitine palmitoyltransferase 1A**	773	LRGRGPLM
	24429592	**Chondroitin beta1,4 N-acetylgalactosaminyltransferase 2**	542	EEFNRGRGLN
	30350206	**AP20 region protein isoform B**	170	KRGRGCNG
	27881700	**Mitochondrial topoisomerase I**	602	KIEPPGLFRG RGDHPKMGML
	13994294	AAA-ATPase TOB3	578	-
Other	2772564	ADP / ATP carrier protein	298	-
	4506457	reticulocalbin	317	-
	27462366	**AG02**	932	GKKRRGRRSKKERRRGRKEG
	7661890	**Sorting nexin 17**	470	SGSTSSPGRG RGEVRLELAF
	19263717	**Similar to RIKEN cDNA 2610027L16 gene**	536	RGRGAKGSG
	7209305	**FLJ00002 protein**	1513	LHRLRGHVAV RGLSKGFGLA
	18379346	**VPS10 domain receptor protein SORCS 3**	1222	AGERRGRGI
	19913532	**Tubby like protein 2**	520	GDGRGERGL
	15076827	**Pcph proto-oncogene protein**	428	VRGKLHQPEE VQRGSFYAFS
	4589624	**KIAA0990 protein**	802	ARGDARGAQL
	17474221	**Similar to KIAA1595 protein**	140	SLARGFSLLR MPKMPELRGK
	24899186	**KIAA2011 protein**	1091	RKTPRGKRGW
	4507285	**Syntaxin 10**	249	RGEVQKAVNT ARGLYQRWCE
	10092621	**Oncostatin M precursor**	252	FPSEETLRGL GRRGFLQTLN
	27436933	**Orthodenticle 2 isoform b; homeobox protein OTX2**	289	SSRGWQGRRG
	23397570	**Hypothetical protein FLJ31579**	304	LSERASRGSF HVSQAILTPR VKTIARGLVG
	27499858	**Hypothetical protein XP_208775**	364	EGRGRDRGRL
	29789407	**Hypothetical protein LOC284996**	400	PGGRGAAPWV ALVARGGCTF
	17484894	**Hypothetical protein XP_066268**	176	GRGPEPSGWE LRRGRCAFGK
	27734689	**Hypothetical protein FLJ36112**	283	ATLERGRGP
	7513003	**Hypothetical protein KIAA0522**	1560	VGPRPPRERG QLSRGASRSS
	30425424	**Hypothetical protein FLJ40434**	334	RGGPVGLLQGRGAAVAEADPLHHDEVRLLRAHGRG
	14150001	**Hypothetical protein DKFZp434G118**	939	SERGLHSPSQ RSHRGPSQRR
	27478389	**Hypothetical protein XP_166555**	166	YLRAAHGRGM ERGLLCVPRR
	8922609	Hypothetical protein FLJ10709	586	-
	30268243	**Hypothetical protein**	1679	FPVSQKRGTI ENERGKPLPS
	29744798	**Similar to hypothetical protein FLJ33516**	1982	YREGLVRGSF RGSFLDYYAA
	23270808	**Similar to hypothetical protein FLJ31614**	740	AITFQEFARG FLGSLRGGRR
	23272713	**Unknown (protein for IMAGE:5722844)**	557	DFTRGSRGRK
	22800407	**Unknown (protein for MGC:15173)**	399	SPRQPRGGRGGGACSAKKERGG
	7022583	**Unnamed protein product**	864	RGERGNDESA
	28070992	**Unnamed protein product**	124	RGPEFD ARPLPTRGCD

TABLE IV
Proteins identified with the ASYM25 aDMA-specific antibody

Function	Accession	Protein	a.a	ASYM25
Pre-mRNA processing	5453840	**RH70 RNA heliase**	650	RGGGFGDRDRDRDRGGFGARGGG
	3915658	**•ATP-dependent RNA heliase A**	1270	RGVSRGGFRGNSGGDYRGPSGGYRGSGGFQRG
	1041747	**•Sam 68**	443	RGRGVPVRGRGAAPPPPPVPRGRGVGPPRGALVRG
Protein translation	35903	Ribosomal protein L7	248	-
	4506671	Ribosomal protein P2	115	-
	4503477	eEF1 beta 2	225	-
DNA transcription	29743861	**Similar to widely-interspaced zinc finger motifs**	957	VLRGGIPGPPLYPGRGRTAF
	13376064	**Zinc finger protein 408**	720	CGRAFRQRGNLRGHLRLHTG
Receptors and signaling	7656967	**Cadherin EGF LAG seven-pass G-type receptor 1**	3014	SVRRGFRGC
	21929089	**Seven transmembrane helix receptor**	1464	RGGQSSARGV
	30156191	**Similar to MrgE G protein-coupled receptor**	418	RVERGPQRPPPRGFPGLILL
	21594833	**Platelet-derived growth factor receptor, beta polypeptide**	1106	EQTVRCRGRG
	28422541	**Phosphatidylserine receptor beta**	441	PPRPGRGALVSGSGLRRGRS
	6760665	**FLASH homolog RIP25**	1982	EDSRRGRKDIRHSQFNRGTE
	22532415	**Regulatory protein NOXO1-gamma / p47 phox**	376	SGTGFRGGDDPAGEARGFPE
	14579311	**Atypical PKC isotype-specific interacting protein long variant b**	1273	RGRGCNESFR
Cytoskeletal proteins	446631	**Collagen:SUBUNIT=alpha2:ISOTYPE=IX**	618	RGGRGHPGMPGPPGIPGLPGRPGQAINGKDGDRGS
	115313	**Collagen alpha 1(V) chain precursor**	1838	RGQRGPTGPRGERGPRGITGKPGPKGNSGPPGERG
	13435369	**Desmocollin 3 isoform Dsc3b preproprotein; desmocollin 4**	839	SCRGAGHHHT LDSCRGGHTE
	18698322	**Synemin**	1251	ELRGRRGREG
DNA repair	4505339	Nibrin	754	-
	5031923	**MRE11**	708	GRGRGRRGGRGQNSASRGGSQRGRA
	5032017	Rad50	1318	-
Enzymes	181362	**Cytochrome P-450 S-mephenytoin 4-hydroxylase**	485	GRGIFPLAERANRGFGIVFS
	2209278	**Oxytocinase splice variant 2**	1011	EYEPRGSRLLVRGLGEHEME
	9966821	**Lysosomal apyrase-like protein 1**	604	HVRGRGDWV
	5305594	**Ca2+-independent phospholipase A2 short isoform**	752	AYMRGMYFRMKDEVFRGSRP
	7435520	**Adenosylhomocysteinase (EC 3.3.1.1) DKFZp564A1523**	797	HRGGSRRGKI
	15026974	**Obscurin Rho GEF**	6620	LGPRGPLGLFRPEPRGASPP
Others	29735143	**Similar to KIAA1311 protein**	949	RGRGRGRGGR
	18916736	**KIAA1944 protein**	657	RRGRGCTLQY
	3694661	**Carrier protein-like; similar to Q01888**	391	RGPAPCRAGPGARHLRPWPESPRPEPRGLPGPGRG
	22328128	**Similar to Meningioma-expressed antigen 6/11 (MEA6) (MEA11)**	811	GGEGRGSRGPGNPLDHQITKERGESSCERL
	11990772	**BA534G20.4 (supervillin)**	1084	RGRGAANDS
	1945155	**MN1 probable tumor suppressor**	1342	GRGRGRRK
	27499679	**Similar to 2210403N09Rik protein**	276	RGHSAGRGDE
	11359992	**Hypothetical protein DKFZp434F117.1**	834	RGRCDSRGNQ
	7657017	**Hypothetical protein DJ328E19.C1.1**	921	KGKKRRGRRSKKERRRGRKE
	20483460	**Hypothetical protein XP_116897**	258	LFRGKAGKPSQGRGMAVRLM
	29738552	**Hypothetical protein XP_061446**	344	RSELQARGLRRGNAGRRLEL
	30154584	**Hypothetical protein XP_302601**	114	QRGRSGSGNFGGGRGGGFSGNDN
	24432043	**Hypothetical protein FLJ13511**	668	RGRGGPESP
	22749001	**Hypothetical protein FLJ30373**	192	MGHEGRGQSGELGDLGARGP
	21732492	**Hypothetical protein**	1289	RGRPEISLDERGEGGHVHTS
	21732438	**Hypothetical protein**	723	RGRGCALQYQ
	13528825	**Similar to hypothetical protein FLJ20003**	316	EGRGRGRD
	21595426	**Unknown (protein for MGC:40478)**	412	KRTKDRGTMDDDDFRRGHPQ
	36575	**Unnamed protein product**	478	SQRGHSRGRN
	14336692	**Unknown**	321	SGRGAGRGGARGFSTVTRGH

Protein Translation

A protein complex that was affinity-purified by SYM10 but not SYM11 was the cleavage and polyadenylation specificity factor (CPSF) complex including the 25-, 68-, and 100-kDa subunits (Table I). The poly(A)-binding proteins PABP1 and PABP2 as well as eIF-4G were purified (53). CPSF6, a protein of 68 kDa, has several RG repeats that are likely the epitope for SYM10, and the other components are likely co-associating proteins (54). PABP1 has been shown to be asymmetrically dimethylated by CARM1 at arginine 455 and 460, which is not an epitope for SYM10 (46). Thus, PABPs and eIF-4G are likely purified as co-associating proteins with the CPSF complex. SYM11 purified the elongation factor 1 δ isoform, which is a guanine nucleotide exchange factor (Table II). ASYM24 and ASYM25 purified several ribosomal proteins and tRNA components (Tables III and IV).

DNA Transcription

The role of arginine methylation in transcriptional regulation is well established with histones being a major target of protein-arginine methyltransferases (see the Introduction). As histones do not contain RG-rich sequences, we did not expect to identify them, and indeed histones were not identified. SYM11 purified transcriptional proteins involved in all aspects of tran-

scription. Several histone-modifying proteins including MYST histone acetyltransferase 2 (Table I) (55) and histone-lysine methyltransferase DOT1L were identified (Table II) (56). Two proteins with methyl-binding domains were identified (Table II) including methyl-CpG-binding protein 2 (MBD2), which plays a role in transcriptional control (57, 58), and the transcription terminator factor I-interacting protein 5. A protein involved in transcriptional elongation, DSIF p160, and a serine phosphatase TFIIF-associated CTD phosphatase I, which is involved in the recycling of RNA polymerase II (59), were purified with SYM11. Several transcription factors were also identified including the basic helix-loop-helix leucine zipper protein TFEB, p98 Rel homolog, interleukin enhancer-binding factor 1 and 2 (ILF1 and ILF2), zinc finger DNA-binding proteins, homeodomain protein PBX4, the C/EBP-induced protein, and ZNP9. Interestingly, ILF2 was identified with SYM11 and ASYM24 suggesting that it contains both modifications. The amino acid sequence of TFEB has RG repeats that are likely candidates for SYM10 recognition. A chromosomal translocation leads to a promoter switching, giving rise to an α*TFEB* fusion gene, which leads to renal cell carcinomas (60, 61). The ZNF9, RING zinc finger protein 9, was identified by LC/MS/MS, and the presence of RG repeats in its sequence suggests that it is a direct epitope of SYM10. The function of ZNF9 is unknown, but it is a nucleic acid-binding protein with an AIR1 (arginine methyltransferase-interacting protein 1) domain (62). The presence of CCTG repeats in intron 1 of *ZNF9* gene leads to myotonic dystrophy type 2 (63). Thus, arginine methylation may regulate the transcriptional initiation, elongation, and termination.

Receptors and Signaling

Six G-coupled receptors were identified with the DMA-specific antibodies. Both the α2CII adrenergic receptor and the urotensin II receptor were purified by SYM10 (Table I); the α-2A adrenergic receptor was identified with SYM11 (Table II), and three unclassified receptors were identified with ASYM25 (Table IV). This analysis is the first indication that G-coupled receptors may contain dimethylated arginines, and the presence of RG repeats suggests that the receptors were directly recognized by the antibodies. The α2CII adrenergic receptor is present in the axon terminals of neurons of spinal cord origin and may mediate nociceptive information (64). The urotensin II receptor has vasoactive properties, and its increased expression in cardiomyocytes correlates with cardiac dysfunction (65).

Other cell surface receptors that were identified include Roundabout 1 (Table II), which is an axon guidance receptor that controls axon crossing of the central nervous system midline (66). The platelet-derived growth factor receptor β was identified with ASYM25, and it contains RG repeats (Table IV). A phosphatidylserine receptor was identified with ASYM25. The phosphatidylserine receptor is required for recognition of the asymmetrical phosphatidylserine distribution of a dying cell (67). A potassium voltage-gated channel was identified with SYM10 (Table I), and a calcium channel was identified with ASYM24 (Table III).

Intracellular proteins involved or associated with signaling proteins were also identified including the Crk-associated substrate p130cas, the breast cancer antiestrogen resistance 3 (BCAR3), the STE20-like kinase from prostate (PSK), ataxia telangiectasia (ATM), protein kinase N β, FLASH homolog RIP25, phosphatidylinositol-4-phosphate 3-kinase, and NOXO1. CAS is an SH3 domain-docking protein that participates in FAK-dependent cell migration (68). BCAR3 contains a putative SH2 domain and a guanine nucleotide exchange activity and has been shown to associate with CAS (69). The absence of RG repeats in CAS and BCAR3 indicates that they were likely co-purified. PSK contains several RG-rich repeats and was identified with SYM11. PSK is known to activate mitogen-activated protein kinase pathways (c-Jun NH (2)-terminal kinase, p38, or extracellular signal-regulated kinase) (70). ATM is a serine kinase that is involved in DNA repair signal transduction; the absence of RG repeats indicates that it was co-purified (71). Protein kinase N β is an unassigned kinase identified with ASYM24. The FLASH homolog RIP25 is known to be involved in signaling to the interleukin 2 gene expression and was identified with ASYM25. Phosphatidylinositol-4-phosphate 3-kinase C2 β represents a class II PI3K and has been shown to be recruited to tyrosine kinase complexes (72). NOXO1 γ is an RG-containing protein purified with ASYM24 (Table III). NOXO1 was identified as a homolog of p47phox and has been shown to regulate superoxide production (73).

Several proteins implicated in apoptosis were also obtained including HtrA2, apoptosis inhibitor protein (IAP), and mortalin-2 (74). These proteins are co-purified as they are devoid of RG repeats. The identification of the phosphatidylserine receptor with ASYM25 suggests that arginine methylation may play a role in cell death.

DNA Repair

The MRE11-Rad50-NBS1 complex (75) was purified by using ASYM25. The MRE11-Rad50-NBS1 complex localizes to radiation-induced foci upon double strand DNA breaks (76). The presence of RG repeats in MRE11 suggests that MRE11 harbors an epitope for ASYM25 and that Rad50, NBS1, and ATM are co-purifying proteins with ASYM25 and SYM11.

Cytoskeleton

Many cytoskeletal proteins including keratin type II cytoskeletal 1, α 1 type VII, XI, and XXII collagen precursors, spectrin, ankyrin, and dynein were purified (Tables I–IV). It is tempting to speculate that some of the components like keratin are contaminants, but the presence of abundant RG repeats makes these proteins likely targets for the DMA-specific antibodies.

DISCUSSION

In the present study we have identified over 200 proteins that contain RG-rich repeats and are putative substrates of protein-arginine methyltransferases. The identification and purification of proteins known to contain dimethylated arginines such as SmB/B′, SmD1/D3, p80-coilin, EWS, TLS, and Sam68 confirm our strategy. Most of these proteins are associated with RNA metabolism and contain RG-rich motifs. Gary and Clarke (1) searched for PRMT substrates by using databases with glycine-arginine-rich consensus sequences. The glycine-arginine-rich consensus sequence was derived from the methylation sites of fibrillarin, nucleolin, hnRNP A1, basic fibroblast growth factor, and MBP (1). Putative substrates identified included mammalian EWS, hTAFII68, hnRNPs, TLS, and ribosomal S2. Thus, the major category of putative substrates was RNA-binding proteins. By using antibodies that are specific for sDMA or aDMA, we were able to minimize the amount of RNA-binding proteins identified. Each antibody was designed with unique surrounding sequences; thus, we were able to identify proteins that could not be predicted by using a glycine-arginine-rich domain search.

The presence of Sm proteins and p80-coilin in SYM10 and SYM11 immunoprecipitations indicates the specificity of these antibodies for sDMA-containing proteins in cellular lysates. It has been reported that the abundant hnRNPs account for close to 65% of all arginine methylation that occurs in the cell (19). These proteins often contain long stretches of RG and RGG repeats that have been demonstrated to contain asymmetrically dimethylated arginines. The fact that the SYM10 and SYM11 immunoprecipitated only a few hnRNPs further demonstrates the specificity of the antibody for sDMA and not aDMA or non-methylated RG-rich proteins. We would have expected the ASYM24 and ASYM25 antibodies to recognize the spectrum of hnRNP proteins as well as the nucleolar proteins fibrillarin, nucleolin, and ribosomal protein S2. In addition, we have not identified the cell death regulator aven (77) and 53BP1 (78), two proteins known to contain extensive RG motifs. This demonstrates that the methyl antibodies that we generated recognize a subset of methylated proteins excluding the majority of hnRNPs. Actually, methylated peptides corresponding to hnRNP K arginine-glycine repeats were not recognized by ASYM24 in an enzyme-linked immunosorbent assay (26). In addition the mass spectrometry data did not identify the SMN and PRMT5 complexes in the SYM10 immunoprecipitations performed with HeLa-S3 cells. SMN and PRMT5 were readily identified in SYM10 immunoprecipitations in T4 HeLa as shown previously (27). It has been shown that SMN complexes behave differently in certain HeLa cell types (79).

Thus, the proteins that were identified through the large scale immunoprecipitation can only be present if 1) they contain DMA and are directly immunoprecipitated by the antibody, 2) they are present in a complex that contains DMA, or 3) they are contaminating proteins that were nonspecifically bound during the purification process. The presence of potential epitopes (RG repeats) suggests that most of the proteins were directly recognized by the methylarginine-specific antibodies. It is possible that some of the cytoskeletal proteins are contaminants, especially keratin, but the presence of RG repeats in their sequences provides a reason for their selective purification. The identification of many proteins in the same complex, such as the pre-mRNA complex and polyadenylation complex, allows us to conclude that DMA is potentially involved in regulating the function of that complex.

The identification of proteins involved in pre-mRNA splicing and transcription was expected (2). The identification of the G-coupled receptors, the polyadenylation complex, and proteins involved in DNA repair and cell death was unexpected. The role of sDMA in snRNP assembly is known and has been proposed as a signal for targeting to the SMN complex (31). Treatment of cell extracts with SYM10 or with methylase inhibitors impairs pre-mRNA splicing (26). The identification of several splicing factors including KSRP and the SR proteins suggests a new level of regulation of splicing by arginine methylation.

The role of arginine methylation in transcription has been known since the identification of CARM1 as a coactivator (6). The regulation in most of those studies involves the histone-arginine methylation. The identification of transcription factors including TFEB, ILF proteins, and PBX as well as the CTD phosphatase, DSIF p160 transcription elongation factor, and the transcription terminator-interacting protein 5 will direct us toward new modes of transcription regulated by arginine methylation. The interplay between sDMA and transcription has been suggested (80); however, the substrates of PRMT5 were not identified. Finally, arginine methylation could also delineate the transition between promoter binding and transcription elongation, an example of which has been demonstrated for SPT5 (81).

The presence of six G-coupled receptors in DMA-specific immunoprecipitations leads us to believe that these proteins are likely to be dimethylated on arginines. The presence of RG-rich sequences further provides evidence that this will be the case. It will be interesting to determine whether the methylation interferes with the signaling or potentially with the recycling/desensitization of the receptors.

The purification of the cleavage- and polyadenylation-specific complex by SYM10 demonstrates that a protein within the complex contains sDMA (54). The presence of RG repeats in CPSF6, the 68-kDa subunit, indicates that this protein is putatively methylated by type II PRMTs. The fact that SMN associates with sDMA-containing proteins (31) and has been dubbed the “master” assembler (82) suggests that the CPSF complex formation, localization, and function may be regulated by SMN complex and/or the methylosome.

The MRE11-Rad50-NBS1 complex (75) was purified by using ASYM25. In addition, the ATM kinase was purified with

SYM11. The MRE11-Rad50-NBS1 complex localizes to radiation-induced foci upon double strand DNA breaks (76), and the ATM kinase is involved in signaling DNA damage (71). The presence of RG repeats in MRE11 suggests that MRE11 harbors an epitope for ASYM25 and that Rad50, NBS, and ATM are co-purifying proteins. The purification of proteins involved in DNA repair indicates that arginine methylation will have a role in this cellular process. Arginine methylation may regulate the signaling of DNA damage, protein-protein interactions, or protein localization during DNA damage.

In conclusion, the identification of proteins containing DMA will allow us to shed some light on new roles for this post-translational modification. The methylation of every protein on the list of putative methylated proteins will have to be confirmed, and their physiological roles will have to be demonstrated. The present study will facilitate the integration of arginine methylation in pre-mRNA splicing, protein translation, receptor signaling, transcription, DNA repair, and the cytoskeleton.

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