Research paper

Generation of polyclonal antiserum for the detection of methylarginine proteins

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Abstract

This report describes an approach for the study of the biology of methylarginine proteins based on the generation of immunological reagents capable of recognizing the methylarginine status of cellular proteins. Two forms of an immunizing peptide were prepared based upon an amino acid sequence motif found most prevalently among verified dimethylarginine-containing proteins. One form of the peptide was constructed with 7 arginine residues alternating with 8 glycine residues. None of the arginines used in the synthesis were methylated. The alternative form of the peptide was synthesized with the identical repeating GRG sequence, but with asymmetrical dimethylarginine at each arginine residue. A methylarginine-specific antiserum was generated using the latter peptide. ELISA and western blotting of glycine arginine-rich peptides, each synthesized with or without asymmetric dimethylarginine, demonstrate the methyl specificity of the antiserum. The methylarginine-specific antibody co-localizes with the highly methylated native nucleolin protein conspicuously concentrated in the nucleolus. The methylarginine-specific antiserum recognizes a GRG peptide and bacterially expressed RBP16 only after incubation of the peptide or RBP16 with recombinant protein arginine methyltransferase 1, or cell extracts, respectively. Proteins isolated from cells in different developmental states exhibit different patterns of reactivity observed by western blots. Finally, the methylarginine-specific reagent interacts specifically with the methylarginine of cellular hnRNPA1 and human fragile X mental retardation protein expressed in cultured PC12 cells. An immunological reagent capable of detecting the methylarginine status of cellular methylproteins will facilitate the cellular and molecular analysis of protein arginine methylation in a wide variety of research and biomedical applications.
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1. Introduction

Recent interest in protein arginine methylation has been fostered by the discovery of a growing number of biologically relevant methylarginine proteins (Wada et al., 2002; Boisvert et al., 2003; Bedford and Richard, 2005) and enzymes that are involved in important cellular methylation pathways (Cuthbert et al., 2004; Wang et al., 2004; Miranda et al., 2004; Lee et al., 2005; Birkaya and Aletta, 2005). Protein arginine methylation is capable of providing important regulatory mechanisms for gene expression in a wide variety of biological contexts (Bedford and Richard, 2005). Although numerous examples of "atypical" arginine methylation motifs exist in native proteins (Smith et al., 1999; Xu et al., 2001), there is general agreement that the principal consensus site of arginine methylation for protein arginine methyltransferases typically occurs in glycine arginine-rich domains (Gary and Clarke, 1998; Wada et al., 2002; Boisvert et al., 2003). The RGG "box" motif that is present in many heterogeneous nuclear ribonucleoproteins and other RNA binding proteins is often cited as a consensus site for arginine methylation (Liu and Dreyfuss, 1995; Wada et al., 2002). Data compiled from the increasing number of methylarginine proteins verified by mass spectrometry most frequently generates the minimal consensus sequence of GRG (Rawal et al., 1995; Belyanskaya et al., 2001; Frankel et al., 2002; Miranda et al., 2004).

Advances in the identification and biochemical analysis of cellular methylproteins are hindered by the lack of a simple means of determining the methylation status of native cellular proteins. Mass spectrometry, though precise and authoritative, is a specialized and exacting approach to characterization that requires sophisticated and expensive instrumentation. Metabolic radiolabeling of methylproteins can be problematic due to a variety of obscure kinetic parameters that are potentially affected by alterations in protein synthesis, equilibria across sub-cellular compartments and enzyme activity. Stable isotope labeling of methylproteins, which utilizes cells in culture treated with methionine composed of a methyl group that consists of carbon-13 and three deuterium atoms, is a reliable new approach to methylprotein identification, but still requires the generation of mass spectra for analysis (Ong et al., 2004). Antibodies raised against two different symmetrical dimethylarginine peptides and asymmetrical dimethylarginine peptides derived from SAM68 and nucleolin (Boisvert et al., 2002; Boisvert et al., 2003; Cote et al., 2003) offer, instead, a relatively simple approach to methylprotein analysis and identification by immuno-logical approaches. Immunodetection methods are common to most research laboratories and, in the case of anti-phosphotyrosine antibodies (Glenney et al., 1988), have fostered rapid discovery and analysis of an important category of signal transduction molecules.

The purpose of the present work is the demonstration of a general methylarginine-specific antibody derived from immunization with a poly-GRG peptide harboring asymmetric dimethylarginine at every arginine residue. The immunological reagent, designated anti-mRG, is specific for the methylated peptide and does not react with the same peptide sequence containing non-methylated arginine residues by ELISA or western blot. Anti-mRG co-localizes in situ with the methylprotein, nucleolin, by immunocytochemical staining. Further tests of methyl specificity include demonstrations of anti-mRG binding to recombinant protein or peptides containing GRG only after in vitro methylation by protein arginine methyltransferase 1 (PRMT1). Methylproteins isolated from cells in different developmental states also react differentially with anti-mRG in immunoprecipitation and western blot assays. Neurotrophin-mediated increases in the protein methylation of native hnRNPA1 or human fragile X mental retardation protein expressed in PC12 cells can also be detected by anti-mRG. The utility of this novel reagent should facilitate research on protein methylation by increasing both the speed and ease of methylprotein identification and the determination of the methylation status of methylproteins.

2. Materials and methods

2.1. Peptide synthesis

Two peptide amides were synthesized by solid phase method using Fmoc chemistry (see Appendix). The sequences of the peptides were identical except for the presence of asymmetric dimethylated arginine in one peptide versus unmodified arginine at the corresponding sites in the alternative peptide. The sequences of the peptides were as follows; the asymmetric dimethylarginine-containing peptide, mRG, H-CG**R**G**R**G**R**G **R**G**R**G**R**G**R**G-NH$_2$ (where **R** indicates N^ω,N^ω-dimethylarginine) and the non-modified arginine-containing peptide, RG, H-CGRGRGRGRGRGRGRG-NH$_2$. Two additional peptides derived from the primary amino acid sequence of nucleolin were synthesized as follows; Ac-GRGGF GGRGGFRGGRGG-NH$_2$ and Ac-G**R**GGFGG**R**G GF**R**GG**R**GG-NH$_2$ (where **R** indicates N^ω,N^ω-dimethylarginine). Automated chain assemblies of the peptide amides, 0.1 mmol scale each, were carried out in DMF on a continuous-flow Millipore model 9050

peptide synthesizer using Sieber amide resin (0.16 mmol NH_2-groups per one gram of resin). The side-chain protecting groups used were: Pmc for Arg and Mts for R (Szekely et al., 1999). Fmoc-removal was accomplished with 25% piperidine in DMF. Single couplings were carried out for 1.5 h using in situ protocols with Fmoc-amino acid/TBTU/HOBt/DIEA in ratios of 1/0.9/1/1.8 (0.4 mmol Fmoc-amino acid=4 equivalents). Following chain assembly, the side-chain protected peptide resins were washed with DCM and petroleum ether (fraction 40–60 °C) and dried in vacuo for 24 h.

Cleavage/deprotection cocktails (20 ml resin /g peptide) containing TFA/H_2O/TIPS in ratios of 95/2.5/2.5 were prepared immediately prior to use. The peptide resins were swollen in DCM, filtered and cleavage cocktail was added and capped under N_2. The time for cleavage/deprotection (1 to 5 h) was established according to real time RP HPLC separation of reaction mixture. The peptide mixtures were filtered separately from the resins, washed with 2×2 ml TFA and evaporated in vacuo at room temperature. The peptides were precipitated with 20 volumes cold ethyl ether and then the pellets were separated, washed with ether (3×30 ml) and dried. The peptide, mRG, was additionally deprotected for 45 min with Tms-Br/thioanisol according to the modified procedure of Sparrow and Monera (1996).

Peptides were purified by preparative RP HPLC (Fields et al., 1991) on a Waters RCM 15RP18 column (100×25 mm I.D.) using a linear gradient of acetonitrile (0–35% in 70 min) in the presence of 0.1% TFA (Rivier et al., 1984) and stored as lyophilized powder. The purity and identity of each peptide were confirmed by RP HPLC and ESI MS.

2.2. Preparation of antisera and IgG fractions

Standard methods of immunization, boosting and bleeding of rabbits were carried out by Bethyl Laboratories (Montgomery, Texas). As instructed, 2 mg of the immunizing peptide was covalently conjugated to KLH to increase antigenicity. Approximately 300 µg was reserved for ELISA testing and 200 µg was injected on 8 separate occasions (day 0 and at 2, 4, 6, 8, 10, 14, and 18 weeks later). Bleeds at weeks 5, 7, 9, 11, 13 and 19 were stored under aseptic conditions. ELISA was performed on week 5. Crude sera were processed to produce an IgG-enriched fraction. After centrifugation to remove aggregated proteins (12,000 $\times g$ 5 min), the serum supernatant was diluted with an equal volume of Tris buffer (100 mM; pH 8). The preparation was applied to a Protein A Sepharose column by gravity flow. The column was washed with 10 to 30 ml 100 mM Tris pH

8 until the OD_{280} of eluent fractions fell to ≤0.05. After washing the column with 10 ml 10 mM Tris pH 8, the IgG fraction was eluted with 100 mM glycine pH 3. The IgG elution was collected in 500 µl fractions in 1.5 ml conical tubes containing 50 µl of 1 M Tris pH 8 to neutralize the preparation. Sodium azide was added to a final concentration of 0.02%.

2.3. Processing of cellular proteins for analysis

The cell culture of *Trypanosoma brucei* (procyclic form, clone IsTar1 EATRO164; bloodsteam form, strain 427) has been described previously (Pelletier et al., 2001). Proteins from whole cell extracts of *T. brucei* were prepared by harvesting in 20 mM phosphate buffer (pH 7.9) containing 150 mM NaCl and 20 mM glucose. After centrifugation at 6090 $\times g$, the cells (1.2×10^9 cells) were Dounce homogenized in TE buffer (1 mM Tris pH 8; 1 mM EDTA). The homogenate was centrifuged at 12,000 $\times g$ for 10 min. The supernatants were divided into several equivalent aliquots. SDS-PAGE loading buffer was added to aliquots used for western blotting. Proteins from mitochondrial extracts were prepared according to Pelletier et al. (2001).

PC12 cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 25 U/ml penicillin G, 25 µg/ml streptomycin, 10% donor horse serum, and 5% fetal bovine serum. The induction of neuronal differentiation was initiated by decreasing the serum concentration to 1% donor horse serum with the addition of 50 ng/ml nerve growth factor (NGF) purified from male mouse salivary glands (Mobley et al., 1976). PC12 cell nuclei were isolated from proliferating and NGF-treated cultures. Cultures were first washed with phosphate-buffered saline (PBS; pH 7.5, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) after which the cells were harvested in Tris buffer (20 mM Tris pH 8, 0.1% Triton X-100, 10 µg/ml benzamidine, 1 mM phenylmethylsulfonyl fluoride). After washing the isolated nuclei in the same buffer without detergent, the nuclei were placed in a high salt buffer (20 mM Tris pH 8, 300 mM NaCl, 10 µg/ml benzamidine, 1 mM phenylmethylsulfonyl fluoride) and rotated at 4° for 10 min to extract nucleoplasmic proteins.

2.4. Recombinant proteins

RNA binding protein 16 (RBP16) was expressed as a maltose-binding fusion protein (MBP-RBP16) in DH5α *Escherichia coli* and purified by amylose and polyU-Sepharose chromatography as previously described (Hayman and Read, 1999). Rat PRMT1 was expressed

as a glutathione *S*-transferase (GST) fusion protein from pGEX-PRMT1. BL-21 cells were used to express the pGEX-PRMT1. After growth in LB medium at 37° to an optical density of 0.6, induction was carried out with 0.1 mM isopropyl β-D-thiogalactopyranoside for 2 h and harvested by centrifugation at 5000 ×g for 10 min at 4°. Cells were re-suspended in PBS containing 10 μg/ml benzamidine and 1 mM phenylmethylsulfonyl fluoride for lysis by sonication on ice. The sonicate was centrifuged at 14,000 ×g for 20 min at 4°. The supernatant was mixed with glutathione-Sepharose 4B for 10 min at room temperature and the mixture diluted with PBS for loading onto a mini-column. The column was washed with 30 volumes of PBS and the GST-PRMT1 fusion protein was eluted with PBS containing 5 mM reduced glutathione.

2.5. In vitro methylation

In vitro methyltransferase reactions were performed at 36 °C for 1 h in 80 mM Tris pH 8. The reaction vessel contained 100 μM *S*-adenosylmethionine (SAM), the methyl donor and protease inhibitors (1 μM phenylmethylsulfonyl fluoride and 10 μM benzamidine). Methylation reactions were stopped by adding 3× SDS-PAGE sample buffer (187.5 mM Tris buffer, pH 6.8; 30% glycerol; 6% SDS; 7.5 mM EDTA; 7.5 mM EGTA; 0.003% bromphenol blue) and heated for 5 min in a boiling water bath.

2.6. Immunoblots

Western blots were performed using standard SDS-PAGE and electrophoretic transfers. In most experiments, PVDF membranes were used. Membranes were incubated in Tris-buffered saline (TBS, 20 mM Tris pH 7.6, 137 mM NaCl) blocking solution containing 5% non-fat milk for 1 h at room temperature and then incubated with primary antibody for 1 h at room temperature. After extensive washing with TBS containing 0.1% Tween-20, membranes were incubated with horseradish peroxidase coupled anti-mouse or anti-rabbit IgG for 1 h at room temperature and subsequently washed three times in TBS containing 0.1% Tween-20. Protein bands were visualized with Pierce Supersignal kit (IL, USA). In the case of anti-FMRP and the corresponding anti-mRG staining, nitrocellulose membrane was employed with 1 h blocking in PBS containing 3% milk. Primary antibodies were incubated with the membrane overnight at 4° and developed in a similar manner as the other blots. Mouse monoclonal anti-hnRNPA1 was kindly provided by Gideon Dreyfuss. Rabbit polyclonal anti-FMRP and mAB-2160, used to detect human FMRP, were obtained from Chemicon.

2.7. Immunoprecipitation

Cells were washed twice with ice-cold PBS. Cell lysates were collected in 100 μl (PBS + 1% SDS) and then held in a boiling water bath for 5 min. Nine volumes of ice-cold buffer (10 mM Tris buffer pH 7.4, 100 mM NaCl and 2.5 mM MgCl$_2$) containing 1% Empigen, 1 μM PMSF, 1 mM EDTA and 10 μg/ml benzamidine was added to each sample. Samples were incubated on ice for 10 min and centrifuged for 15 min (100,000 ×g.) Supernatants were collected and pre-cleared with 6 mg protein A Sepharose for 60 min. The antibody reagent was added for 1 h at 4 °C followed by the addition of 5 mg protein A Sepharose at 4 °C for 1 h. Protein A Sepharose beads with antigen–antibody complexes were washed three times with 1% Triton prior to heating in the presence of SDS sample buffer for 5 min. The eluted material was committed to SDS-PAGE and western blot as described above.

2.8. Immunocytochemistry

Rat PC12 cells were cultured for 48 h on collagen- and polylysine-coated glass coverslips prior to processing for confocal imaging. Following fixation with ice-cold methanol for 10 min, the coverslips were incubated with anti-mRG (1/1000) and anti-nucleolin (1/500; Santa Cruz Biotechnology). The detection of antibody staining was accomplished using Alexa Fluor 488 (Ex 495/Em 519) anti-mouse IgG and Alexa Fluor 594 (Ex 590/Em 617) anti-rabbit IgG secondary antibodies. The cells were observed and images collected with a Biorad MRC 1024 three channel laser scanning microscope. Laser gain and offset were chosen such that the fluorescence of each of the Alexa Fluor dyes was visible only in the proper filter set to eliminate the possibility of crossover when preparing the merged images.

3. Results

3.1. Generation of a methylarginine-specific immunological probe that can be used to identify the methylation status of proteins

Based on inference from a significant number of validated asymmetric dimethylarginine proteins, the tri-amino acid sequence, GRG, is the minimal consensus arginine methylation motif found in most methylarginine proteins. To facilitate the detection of the methylation

status in known methylproteins and the identification of methylarginine proteins, two forms of a peptide for rabbit immunizations were prepared based upon this most common amino acid sequence derived from verified dimethylarginine-containing proteins. One form of the peptide was constructed with 7 arginine residues alternating with 8 glycine residues. None of the arginines used in the synthesis were methylated. The alternative form of the peptide was synthesized with the identical repeating GRG sequence, but with asymmetric dimethylarginines used at each and every arginine residue. Each of the two different peptides (repeating GRG or GmRG) was injected into two rabbits for the production of antisera.

The resulting antisera (anti-RG and anti-mRG) exhibit dramatically different protein recognition properties. ELISA results from the two sets of rabbit sera demonstrate that the reactivities of the antibodies exhibit significant selectivity against the peptides used as the respective antigens (Table 1). Anti-mRG recognizes the methylarginine peptide (GmRG) at titers 29 to 43 times more dilute than that for the recognition of the GRG peptide. The anti-RG antibody was slightly less reactive against the immunizing GRG peptide. The selectivity of anti-RG towards GRG versus GmRG peptides was only approximately 2-fold greater in the case of antiserum #1, but more than 190-fold more in the case of antiserum #2. Anti-RG is also somewhat less reactive against GRG than anti-mRG is against GmRG. The results demonstrate that the predominant epitope recognized by both of the antisera raised against GmRG peptide is methylarginine.

The initial ELISA observations made from crude sera have been confirmed by additional tests using both crude serum and IgG-purified material. Antisera #2 and #4 were used for the preparation of IgG-purified reagents. Under equivalent western blot conditions, anti-mRG recognizes the methylarginine peptide, but not the non-methylated peptide (Fig. 1A). The converse is true of the anti-RG reagent. Pre-immune sera do not recognize either peptide. Anti-mRG is also capable of distinguishing the methylation of another GRG-containing peptide, nucleolin (Fig. 1B). Methyl-peptides are ten to fifty times more reactive with anti-mRG under these condi-

tions. In several cases, anti-mRG western blots of the cysteine-containing glycine arginine-rich peptides (GRG; GmRG) can yield two low molecular mass immunoreactive bands (e.g. Fig. 1B and C). This phenomenon may be due to dimerization of the peptide during storage and handling.

We have also verified the methylarginine-specificity of anti-mRG by demonstrating reactivity with GRG peptide or a protein substrate following methylation of these substrates. GRG peptide (250 ng) does not bind anti-mRG. After incubation of GRG peptide with the methyl donor, SAM, and PRMT1, however, the peptide clearly binds anti-mRG (Fig. 1C). The immunoreactivity requires SAM. Likewise, recombinant RBP16, a *T. brucei* mitochondrial protein that contains 5 GRG repeats (Pelletier et al., 2001), is poorly recognized by the anti-mRG antiserum. After in vitro methylation of the recombinant RBP16 protein in the presence of SAM and crude cell extracts as a source of protein arginine methyltransferase, anti-mRG binding to the recombinant protein is increased up to 18-fold (Fig. 1D). The cell extracts used do not exhibit any immunoreactive proteins when blotted in the absence of MBP-RBP16 (data not shown). These results are consistent with recent evidence, which demonstrates that 2 of the 5 GRG sites of RBP16 are dimethylated in vivo (Goulah et al., 2006).

Nucleolin is a well-characterized, heavily methylated nucleolar protein (Lischwe et al., 1982; Lapeyre et al., 1986). To determine if anti-mRG is capable of recognizing methylarginine-containing protein in situ, we used methanol-fixed PC12 cells (Fig. 2). Following incubation with anti-mRG (Fig. 2A) and anti-nucleolin (Fig. 2B), confocal images were obtained. The merged images of anti-mRG and anti-nucleolin reveal nucleolar staining by both antibodies (Fig. 2C). Thus amongst several punctate figures stained with anti-mRG, the nucleolus reacts with anti-mRG indicative of the presence of the methylarginine epitope within the nucleolar structure (cf. methyl-nucleolin peptide; Fig. 1B).

When considered together, the experiments presented above indicate that the anti-mRG antiserum reacts with methylarginine in peptides and proteins with relatively little cross-reactivity to non-methylated arginine in similar peptide and protein contexts. Thus, the anti-mRG antibody provides a relatively specific tool for detecting methylarginine-containing proteins.

3.2. Detection of changes in methylarginine proteins during changes in developmental state

Cellular differentiation is associated with the modulation of arginine methylation of proteins in a number

Table 1
ELISA antibody titers versus peptide

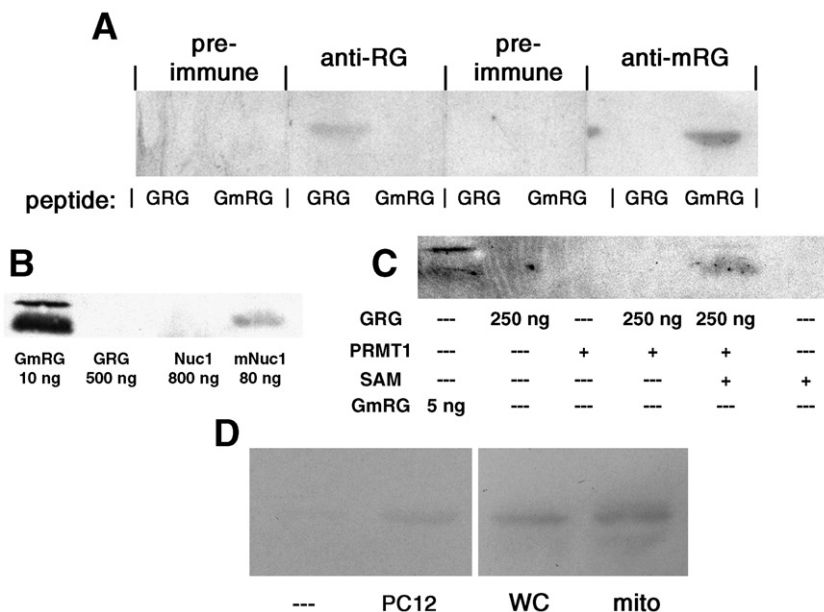| Peptide	Anti-RG	(Rabbit #1 or #2)	Anti-mRG	(Rabbit #3 or #4)
GRG	1/44,600	#1	1/2000	#3
GRG	1/42,800	#2	1/1640	#4
GmRG	1/19,400	#1	1/58,000	#3
GmRG	1/220	#2	1/71,000	#4

Fig. 1. Specificity of antisera raised against GRG and GmRG peptides. (A) Two 16-mer peptides (100 ng) synthesized with repeating glycine–arginine (GRG) or glycine asymmetric dimethylarginine (GmRG) were committed to SDS-PAGE electrophoresis and transferred to western blot membrane for reaction with antisera. Anti-RG antiserum was raised against the non-methylated form of the peptide and anti-mRG against the methylated version of the peptide. Pre-immune sera were taken from all animals prior to immunization with the peptides. Pre-immune sera do not recognize and bind to either form of the peptide. Anti-RG (1/1000 crude serum) recognizes only the unmodified RG peptide. Anti-mRG (1/1000 crude serum) raised against the methylated form of the RG peptide (mRG), likewise, recognizes only the methylated form of the peptide. (B) Asymmetric dimethylarginine peptides, GmRG and mNucl (derived from nucleolin) and the corresponding non-methyl congeners, GRG and Nucl, were committed to SDS-PAGE and western blotting with anti-mRG (IgG-purified; 1/2000). (C) GRG peptide is recognized by anti-mRG only after incubation with recombinant PRMT1 (1 µg) and SAM (100 µM). (D) The mitochondrial RNA binding protein from *T. brucei* (RBP16) was expressed as a fusion with maltose-binding protein (MBP-RBP16). MBP-RBP16 (1 µg) was incubated in Tris buffer (pH 8.0) containing 100 µM SAM and either, no further additions (–), 10 µg of a PC12 cell nuclear extract (PC12), 100 µg of a whole cell extract of *T. brucei* (WC) or 50 µg of a *T. brucei* mitochondrial extract (mito). The reaction tube contents were separated on a 10% SDS-PAGE gel and transferred to PVDF membrane. The membrane was probed with anti-mRG. A barely detectable signal is present in the lane containing RBP plus SAM only. The lane containing PC12 cell extract exhibits a 2.5-fold greater binding of anti-mRG. When *T. brucei* extracts are present in the incubation mixture, the reactivity of anti-GmRG increased 10-fold (WC) and 18-fold (mito).

of different cell types (Cimato et al., 1997; Mowen et al., 2004; Bakker et al., 2004). To examine whether anti-mRG may be of use in the detection of changes in protein methylation as a consequence of developmental events, we employed two different cell model systems. *T. brucei*, the parasitic protozoan responsible for African sleeping sickness, can alternate between the procyclic insect vector form and the mammalian bloodstream
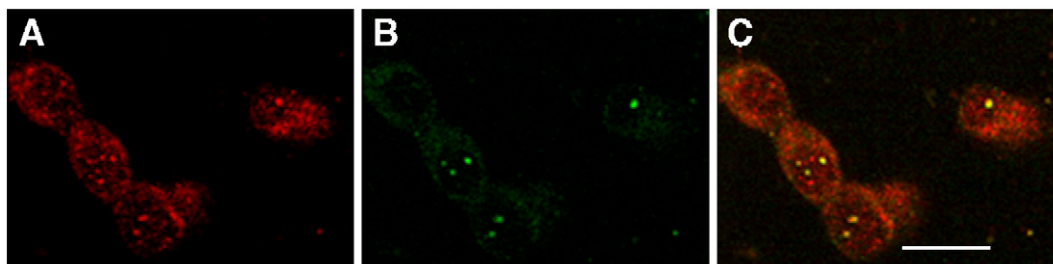

Fig. 2. Co-localization of asymmetric dimethylarginine and nucleolin in cultured cells. Anti-mRG (1/2000) and anti-nucleolin (1/500) antibodies were incubated with methanol-fixed PC12 cells. Fluorescently tagged secondary antibodies against rabbit and mouse IgG were used to detect the locations of asymmetric dimethylarginine and nucleolin, respectively. Panel A, anti-mRG; panel B, anti-nucleolin; panel C, the two images merged. Confocal images were obtained through a 63× oil immersion objective. The white scale bar in panel C is 10 µm.
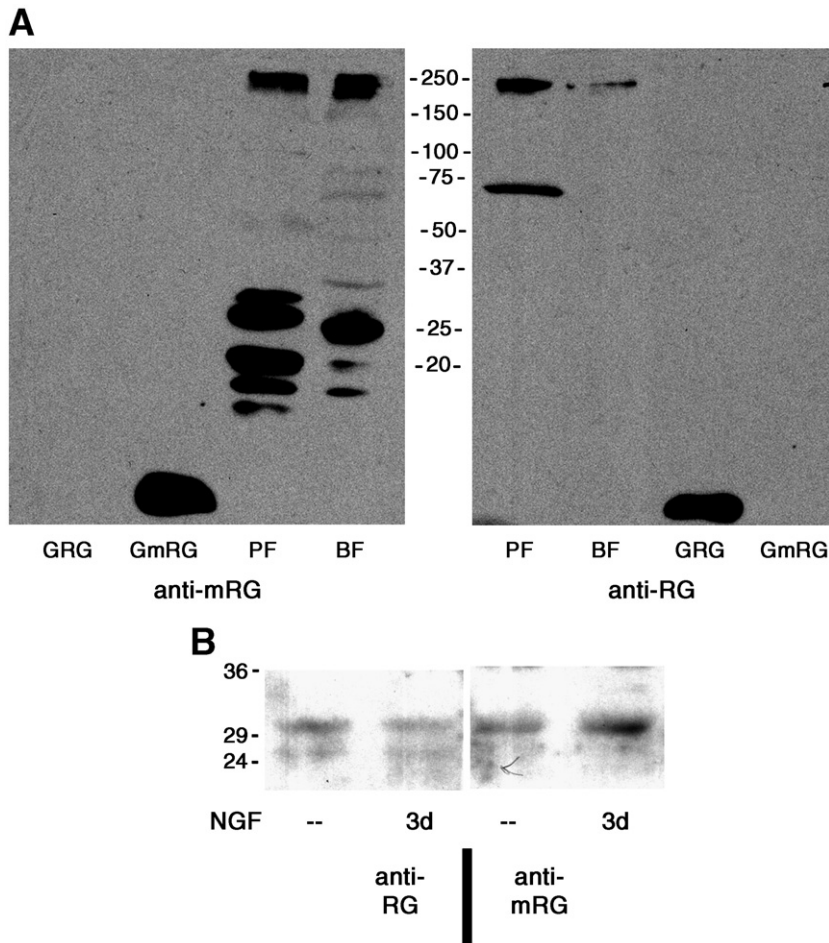
Fig. 3. Differential recognition of cell proteins derived from different developmental states by anti-GRG and anti-GmRG. (A) Equal numbers of *T. brucei* cells of the procyclic (PF) and the bloodstream (BF) forms of the parasite were collected and duplicate aliquots committed to western blot membranes for reaction with anti-mRG (1/5000) and anti-RG (1/5000). The GmRG and GRG peptides (20 ng) were included as positive and negative controls. The electrophoretic migration of molecular mass markers is indicated in kilodaltons between the two membrane images. (B) PC12 cells were cultured without NGF (−) or with NGF for three days (3d). High salt extracts of isolated PC12 cell nuclei were prepared as described in the Materials and methods section and run on SDS-PAGE gels for western blot analysis. Duplicate aliquots of the same protein extracts from −NGF or +NGF samples were loaded in each lane of a 10% SDS-PAGE gel. Separated proteins were transferred to a PVDF membrane and probed with anti-RG (1/1000) and anti-mRG (1/1000).

form of the microorganism. Proteins from whole cell homogenates collected from an equal number of cells in each of the developmental forms were separated by gel electrophoresis and transferred to duplicate western blot membranes for reaction with anti-mRG and anti-RG (Fig. 3A). The anti-mRG blot illustrates a number of immunoreactive protein bands, none of which are recognized by anti-RG. Furthermore, several methylprotein bands derived from the procyclic form of parasites are absent in the bloodstream form and vice versa. These results indicate that anti-mRG recognizes a distinct feature of cellular proteins (i.e. methylarginine) that is not shared with the anti-RG antiserum prepared against

an identical non-methylated peptide. In addition, anti-mRG recognizes several protein species that are unique to either procyclic or bloodstream forms of *T. brucei*.

Nuclear extracts from PC12 cells exhibit several methylarginine proteins that are regulated by nerve growth factor (NGF) treatment (Cimato et al., 1997; 2002). We compared the reactivity of anti-mRG with regard to PC12 cell nuclear proteins by western blotting. One example of a selective increase in anti-mRG reactivity following NGF treatment is the ∼30 kDa protein band illustrated in Fig. 3B. There is also a protein band located at the same position in the duplicate anti-RG blot, but there is no increase in the reactivity

following NGF treatment. These results were replicated in a second independent trial.

These studies, considered together, indicate that fundamental changes in methylarginine protein expression are associated with alterations in developmental state. The results from two rather different cell models capable of undergoing homogeneous developmental changes provide evidence that more extensive analysis of these changes can be useful for the characterization of the protein methylation pathways involved in growth and development.

3.3. Novel uses of anti-mRG to detect neurotrophin-mediated increases in protein methylation

The ability of anti-mRG to recognize methylproteins can be favorably combined with the use of additional antibodies, coupled assays and other novel stratagems for examining the importance of protein methylation in biological systems. This is particularly important because of the potentially large number of immunoreactive methylarginine proteins, the possibility of non-specific antibody cross-reactivity and the difficulty of identifying the immunoreactive forms.

For example, our laboratory is interested in the role of protein methylation in NGF signal transduction. To determine if NGF signaling affects the protein methylation of specific proteins amongst many likely methylarginine proteins, anti-mRG can be used as a reagent to isolate methylarginine proteins by immunoprecipitation. Specific antibody probing of the isolated methylarginine subset of total cell proteins for the presence and/or abundance of suspected methylprotein targets of NGF signaling can be performed in a straightforward manner. Fig. 4 illustrates an

Fig. 5. Correspondence between anti-mRG and anti-FMRP protein staining. Twenty micrograms of total protein from rat PC12 cells (lane 1) or PC12 cells expressing human FMRP (Tf hFMR1; lanes 2 and 3) that were cultured in the absence (lanes 1 and 2) or the presence of NGF for 7 h (lane 3). The western blot membrane was first reacted with anti-mRG (1/1500), the results of which are illustrated in the upper panel. The membrane was stripped and treated with anti-FMRP monoclonal antibody (1/5000) and is shown in the middle panel. The asterisk (*) indicates the migration site of human FMRP, which exhibits increased anti-mRG staining following NGF treatment. The membrane was stripped again and re-probed with anti-HSP70c, the constitutively expressed form of heat shock protein 70 as the protein loading control illustrated in the lower panel.
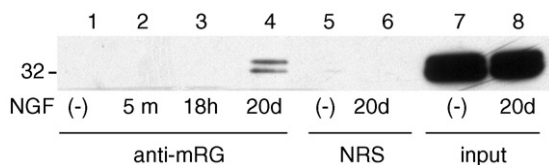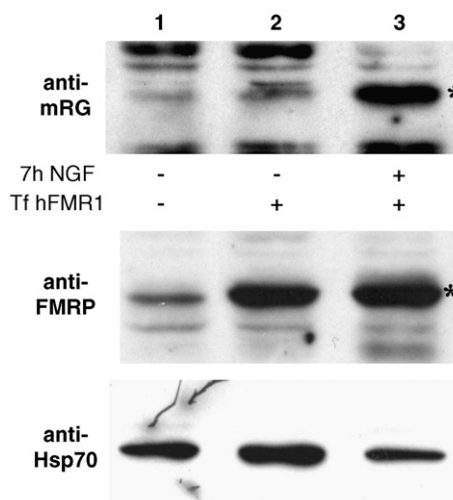

Fig. 4. Long term nerve growth factor-treatment produces increased arginine methylation of hnRNPA1. NGF was added to PC12 cell cultures for 5 min (5m), 18 h (18h), twenty days (20d) or cells were cultured without added NGF (−). Total cell lysates were prepared and the protein loads that were committed to immunoprecipitation from each condition were equalized based on the Biorad protein dye reagent assay. Anti-mRG or IgG from normal rabbit serum (NRS) was added to the samples as indicated. The immunoprecipitated material was collected in SDS sample buffer and run on a 10% SDS-PAGE gel followed by western blot with anti-hnRNPA1. A portion of the equalized starting cell lysates (10%; input) was blotted in lanes 7 and 8 to confirm the presence of equal amounts of hnRNPA1 in the starting material used for the immunoprecipitation.

example of the results from such a strategy. Following immunoprecipitation of PC12 cell proteins with anti-mRG, the immunoprecipitated material was committed to a western blot and probed with an antibody against the methylprotein, hnRNPA1 (Rajpurohit et al., 1994). By this approach, the methylation of cellular hnRNPA1 is below the level of detection by anti-mRG in non-NGF-treated PC12 cells. Short term NGF treatment (minutes to hours) is without effect on anti-mRG reactivity, but neurite-bearing, post-mitotic neuronal differentiated PC12 cells harbor a significant amount of anti-mRG reactive hnRNPA1 after 20 days of NGF treatment. Normal rabbit serum serves as a negative control and the protein input used as the starting material for the immunoprecipitation indicates that the hnRNPA1 present in the (−/+) NGF-treated cells is equivalent. This example demonstrates the utility of this approach for the identification and analysis of cytokine-induced changes in cellular protein methylation.

Recent work has demonstrated that fragile X mental retardation protein (FMRP) undergoes protein methylation, both co-translationally in a rabbit reticulocyte lysate (Dolzhanskaya et al., 2006) and when expressed

in a murine cell line as a Flag-tagged Fmrp (Stetler et al., 2006). As further evidence of the ability of anti-mRG to detect changes in the methylation status of specific methylproteins, we introduced the human FMR1 gene into PC12 cells via transfection (Sung et al., 2003). The overexpression of FMR1 leads to robust expression of human FMRP, detected by western blot of the cell lysates from the transfected cells (Fig. 5, middle panel, lanes 2 and 3). Activation of PRMT1 by NGF stimulation (Cimato et al., 2002) increased anti-mRG reactivity towards the FMRP protein dramatically (Fig. 5, upper panel, lane 3) consistent with the expected increased methylarginine content of the protein.

These studies further indicate that the use of anti-mRG for immunoprecipitations and western blots, when combined with other standard cellular and molecular techniques, offers a practical new tool for studies of protein methylation.

4. Discussion

A growing interest in post-translational modification of proteins by arginine methylation has been sparked by studies linking protein methylation to regulation of signal transduction, transcription, RNA metabolism and protein targeting within cells (Bedford and Richard, 2005). Several methylarginine-specific antibodies have been utilized to successfully identify hundreds of putative arginine methylated proteins (Boisvert et al., 2003). In most cases, the antibody reagents were raised against methyl-peptides derived from confirmed methylarginine proteins; SAM68, SmD3 and nucleolin. An antibody raised against a symmetrical dimethylarginine decapeptide has also previously been described (Boisvert et al., 2002). In the current work, a novel immunological reagent is described based on a 16-mer peptide sequence which harbors asymmetric dimethylarginines in a motif that is widely represented in many methylarginine proteins. The target peptide was designed with two particular characteristics: seven tandem repeats of the minimal arginine methylation consensus motif, GRG, and asymmetric dimethylation at each and every arginine residue. Unlike other antibodies raised against asymmetric dimethylarginine antigens, anti-mRG was generated using the repetitive tri-amino acid consensus motif that is characteristic of many asymmetric dimethylarginine proteins. Previously generated anti-asymmetric dimethylarginine antibodies have instead employed immunizing peptides, the sequences of which were derived from single, specific methylated proteins. In addition, a peptide identical in every respect save for the asymmetric dimethyl-modification of the arginine residues was produced as well. The

latter peptide serves as a control immunogen for the production of antisera that reacts only poorly with methylarginine in peptides and proteins. Both of the antibodies characterized in this work (anti-mRG and anti-RG) add to the tools available in protein methylation research.

The anti-mRG rabbit polyclonal antibody described in this report is expected to be useful for the analysis of asymmetric dimethylarginine proteins such as the heterogeneous nuclear ribonucleoproteins (Liu and Dreyfuss, 1995). Preliminary experiments with Y12-reactive SmD3 (cf. Boisvert et al., 2002; Hebert et al., 2002) indicate that anti-mRG does not react with symmetrically dimethylated arginine proteins (unpublished results). The number of methylarginine residues, the symmetrical or asymmetrical configuration of the dimethylarginine epitope and peptide length of the immunogen may contribute to the protein recognition profile exhibited by the particular antibody preparations. For example, the SYM10 reagent (Boisvert et al., 2002) raised against a decapeptide containing 4 symmetric dimethylarginines was poorly reactive with the same decapeptide harboring only a single symmetrically dimethylarginine or a peptide derived from myelin basic protein with a single symmetric dimethylarginine. Despite the specificity of antibody preparations for symmetric and asymmetric dimethylarginine, the methylation status of any protein that reacts with a methylarginine-specific antibody must be confirmed by other means.

Anti-mRG may be generally useful in two broad applications. First, with regard to the detection of known methylproteins, the antibody can be used to detect changes in the relative degree of methylation. Second, anti-mRG provides another experimental approach to the identification of undisclosed methylarginine proteins. In the latter case, the use of the anti-RG reagent can serve as a useful control. For example, there are a number of anti-mRG-reactive proteins that are uniquely present in either the procyclic or bloodstream forms of trypanosomes, none of which react with anti-RG (Fig. 3A). In the case of PC12 cells undergoing neuronal differentiation, there is a ~30 kDa protein band that reacts more strongly with anti-mRG than with anti-RG in protein lysates from NGF-treated cells (Fig. 3B). Based on the findings of our experiments, the use of anti-RG, an immunological reagent prepared against the identical primary peptide sequence as anti-mRG but without any methylmodification, serves as a suitable means of controlling for cross-reactivity to non-methylarginine epitopes.

The potential involvement of methylarginine proteins in human disease is quite broad (Bedford and Richard, 2005). Several neurodevelopmental diseases arise from gene mutations that implicate methylarginine proteins in the support of normal neuronal functions. Deletion or loss

of function mutations of the survival motor neuron gene 1 (*SMN1*) result in spinal muscular atrophy (Lefebvre et al., 1995). SMN protein binds preferentially to symmetric dimethylarginine proteins important for the assembly and processing of specific RNA-protein complexes (Friesen et al., 2001). Fragile X syndrome results from dynamic mutation, trinucleotide repeat expansion of the fragile X mental retardation gene 1 (*FMR1*). Repression of *FMR1* due to the expanded CGG repeat leads to a decrease of *FMR1* product, the fragile X mental retardation protein (FMRP). Stetler et al. (2006) have recently demonstrated the presence of asymmetric dimethylarginine in Flag-tagged FMRP that was immunoprecipitated from mammalian cells. The dimerization and recruitment of FMRP into large stress granules is, furthermore, methylation-dependent (Dolzhanskaya et al., 2006). Various mutations in *MECP2* are responsible for Rett syndrome, one of the most common causes of mental retardation in females. Recombinant MeCP2 protein exhibits three GRG methylation consensus sites, is an excellent in vitro substrate for PRMT1 methylation and the native protein immunoprecipitated from cell lysates is recognized by anti-mRG (unpublished observations, Aletta lab). MeCP2 is a methyl CpG binding protein that acts as a DNA silencer. Thus, all of these neurodevelopmental genes that are associated with methylarginine modification may conceivably produce abnormal neuronal gene expression when disease-causing mutations are present.

Further experiments will be required to define the role of asymmetric dimethylarginine protein modification in developmental events and to understand better the neurotrophin-stimulated effects on protein methylation. Future work to accomplish these goals will be facilitated by the use of anti-mRG and other methylarginine-specific antibodies.

Acknowledgements

Appendix A. List of abbreviations

Abbreviations used for amino acids and peptides follow the rules of UPAC-IUB Commission of Biochemical Nomenclature in *Eur. J. Biochem.,* 138,9–37 (1984), together with their correction in *Eur. J. Biochem.,* 152,1 (1985); http://www.chem.qmw.ac.uk/iupac/AminoAcid/. Amino acid symbols denote the L-configuration. All solvent ratio and percentages are volume/volume unless stated otherwise.

R Dma, N^ω,N^ω-dimethylarginine; DIEA, *N*-diisopropylethyamine; DMF, *N,N*-dimethylformamide; ESI MS, electrospray ionization mass spectrometry; Fmoc, fluoren-9-yl-methyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MeCN, acetonitrile; Mts, 2,4,6-trimethy-1-sulfonyl, mesitylene-2-sulfonyl; Pmc-2,2,5,7,8-pentamethychroman-6-sulfonyl; RP, reverse phase; TBTU, *N*-[(1-*H*-benzotriazol-1-yl)(dimethylamine) methylene]-*N*-methylmethan aminium tetrafluoroborate *N*-oxide; previously named 0-(benzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; TMS-Br, trimethysilyl bromide.

1. GRG H-CGRGRGRGRGRGRGRG-NH₂
2. GmRG H-CG**R**GR**G**R**G**R**G**R**G**R**G**R**G**R**G-NH₂
3. Nucl Ac-GRGGFGGRGGFRGGRGG-NH₂
4. mNucl Ac-G**R**GGFGG**R**GGF**R**GG**R**GG-NH₂

1. t_R=26.8, purity=69/98 (crude/purified)
2. t_R=27.7, purity=78/99 (crude/purified)
3. t_R=24.4, purity=89/97 (crude/purified)
4. t_R=25.7, purity=91/98 (crude/purified)

RP HPLC was performed on Zorbax 300 5RP18 (150×4.6 mm I.D.) column on Gilson liquid chromatograph, using 0.1% TFA in water in the mobile phase, and a gradient of 0 to 60% 0.1% TFA in MeCN over 60 min. Detection at 214 nm. t_R — retention time.

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