Journal of Neuroscience Research 67:435–442 (2002)
DOI 10.1002/jnr.10123

Nerve Growth Factor-Mediated Increases in Protein Methylation Occur Predominantly at Type I Arginine Methylation Sites and Involve Protein Arginine Methyltransferase 1

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Nerve growth factor (NGF)-specific signal transduction leads to changes in protein methylation during neuronal differentiation of PC12 cells (Cimato et al. [1997] J. Cell Biol. 138:1089–1103). In the present work, we demonstrate that, among NGF-regulated proteins, arginine methylation is more prevalent than carboxylmethylation. Type I protein arginine methyltransferase (PRMT) activity produces asymmetric dimethylation of the terminal guanidinonitrogen of arginines in substrate proteins, particularly glycine and arginine-rich (GAR) segments of proteins. Several GAR peptides were used to assay for methyltransferase activity and to compete with endogenous cellular proteins for the PRMT activity in PC12 cell extracts. Peptides derived from fibrillarin and nucleolin, as well as a synthetic GAR peptide containing a repetitive GRG motif, are each extremely effective at blocking in vitro methylation of the NGF-regulated PC12 cell methylated proteins. Myelin basic protein, a substrate for type II PRMT, selectively inhibits a 45 kDa protein but is a much less effective inhibitor of total methylation at an equimolar concentration. In addition, the fibrillarin- and nucleolin-derived peptides were used to detect elevated PRMT activity in homogenates of NGF-treated PC12 cells. Finally, immunoprecipitation of PRMT1 from PC12 cells provides the first demonstration of an NGF-activated methyltransferase and implicates PRMT1 in NGF signal transduction.

Key words: protein arginine methyltransferase; neuronal differentiation; PC12 cells; carboxylmethylation; N-methylation

Protein methylation is accomplished by specific enzymes that transfer the methyl group of S-adenosylmethionine (SAM) to basic amino acids via N-methylation of the side-chain nitrogens or to the carboxyl groups of acidic amino acids by esterification (Paik and Kim, 1990). Although these modifications were described more than 30 years ago, the cellular consequences of protein methylation are not understood. The possible functional roles of these molecular modifications and the characterization of the enzymes involved have been examined in recent reviews (Gary and Clarke, 1998; Aletta et al., 1998). Among the potential functions of protein methylation is regulation of gene expression during cellular differentiation (Benveniste et al., 1995).

The PC12 cell model is a useful experimental system for addressing the regulatory role of protein methylation in neuronal differentiation. Nerve growth factor (NGF)-specific changes in the methylation pattern of PC12 cell proteins indicate that posttranslational methylation is involved in neurite outgrowth (Cimato et al., 1997). Previously described NGF-mediated regulation of Ras in a methylation-dependent manner may contribute to the biological actions of NGF (Qiu and Green, 1991). The enzyme responsible for carboxylmethylation of Ras is prenylprotein methyltransferase. Protein arginine methylation represents another class of protein methylation that is also likely to play a role in signal transduction (Aletta et al., 1998). The two principal categories of protein arginine methyltransferases (PRMTs) are type I and type II PRMT. Both types of enzyme catalyze the formation of ω-monomethylarginine in proteins. Type I also produces asymmetric N^{ω},N^{ω}-dimethylarginine, whereas type II forms symmetric $N^{\omega},N^{\omega\prime}$-dimethylarginine.

The family of PRMTs has expanded rapidly following the cloning of PRMT1 (Lin et al., 1996). PRMT3, like PRMT1, is a type I enzyme, although the cellular, molecular, and regulatory properties are quite different (Tang et al., 1998). CARM1 was recently described as a coactivator-associated arginine methyltransferase (Chen et

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Received 24 September 2001; Accepted 18 October 2001

al., 1999), but the category of methylation catalyzed by CARM1 has not been formally characterized. Finally, PRMT5 (also known as *JBP1*) and the yeast homologs may be type II PRMTs based on the ability of the expressed protein to methylate myelin basic protein (Pollack et al., 1999). The number of proteins unequivocally verified as type I, asymmetrically dimethylated arginine proteins is growing. In addition to $hnRNPA_1$, nucleolin, and fibrillarin (Gary and Clarke, 1998), PABPII (Smith et al., 1999) contains asymmetric dimethylarginine. Native RBP16 also contains dimethylarginines of the type I variety (Pelletier et al., 2001). SAM68 (Bedford et al., 2000), interleukin enhancer binding factor 3 and nuclear factor 90 (Tang et al., 2000), and Stat1 (Mowen et al., 2001) are good in vitro substrates for PRMT1. On the other hand, symmetrical dimethylation of arginine residues in proteins was, until recently, verified by peptide sequencing and mass spectrometry in myelin basic protein only. Recently, Brahms et al. (2000) have expanded this category to include the spliceosomal Sm proteins D1 and D3.

This study further characterizes the NGF-regulated protein methylation that occurs in cells undergoing neuronal differentiation. We report here that, with regard to NGF-regulated signaling, N-methylation of proteins is more frequent than carboxymethylation in whole-cell extracts. The findings indicate that protein arginine methylation accounts for most of the NGF-regulated increases in protein methylation. In addition, we conclude that NGF regulates type I PRMT activity based on several lines of evidence. NGF leads to activation of endogenous PRMT activity as judged by increased methylation of two peptide substrates derived from known type I proteins. It is also clear that there is an abundance of type I proteins subject to NGF regulation, because the methylation of most NGF-regulated proteins is reduced in the presence of peptides derived from verified type I protein substrates. Finally, consistent with these observations, NGF leads to activation of PRMT1, a type I enzyme.

MATERIALS AND METHODS

Cell Culture

β-NGF was purified from male mouse submaxillary glands, and PC12 cells were cultured as previously described (Cimato et al., 1997). Prior to all experimental treatments, the serum content of the cultures was reduced to 1% for at least 16 hr.

Assay for Methylesterified Proteins

Methylproteins were metabolically radiolabeled as previously described (Cimato et al., 1997). Proteins in whole-cell lysates were separated by SDS-PAGE gradient gels (7.5–15%). Individual lanes were cut into 2 mm sections from 14 kDa to 205 kDa. The presence of methyl esters was detected by base hydrolysis-induced formation of ^{3}H-methanol (Chelsky et al., 1984).

Peptides

Two peptide amides were synthesized by solid-phase Fmoc chemistry. One of the peptide sequences (Ac-GRGGFGGRGGFRGGRGG-NH_2) corresponds to amino acid residues 676–692 of human nucleolin. The sequence of an artificial peptide, designated *GRG,* is H-CGRGRGRGRGR-GRGRG-NH_2. The purity and identity of each peptide were confirmed by RP HPLC/ESI MS. R3 (Ac-GGRGGFGGR-GGFGGRGGFG-NH_2), which is based on a known dimethylarginine motif in fibrillarin, was a generous gift from Dr. Aswad (University of California, Irvine).

Subcellular Fractionation

A cytoplasmic fraction was prepared by harvesting PC12 cells in 20 mM Tris buffer, pH 8, containing 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml benzamidine, and 0.1% Triton X-100. After centrifugation (500*g,* 5 min, 4°C) to remove nuclei, the supernatant was subjected to a second centrifugation (12,000*g,* 5 min, 4°C) to give the cytoplasmic fraction. To generate a nuclear extract, nuclei were collected, washed in Tris, and resuspended in buffer containing protease inhibitors and 300 mM NaCl. After gentle agitation for 10 min, nucleoplasmic extract was separated from insoluble nuclear matrix material (12,000*g,* 5 min, 4°C). In several initial experiments an alternative method for generating subcellular fractions was used (Cimato et al., 1997). Protein was determined with the Bio-Rad assay (Hercules, CA).

Immunoprecipitation and Enzyme Assays

PC12 cell cultures were rinsed and harvested in PBS (4°C) containing 2 mM PMSF and 10 μM benzamidine. After sonication (10% duty cycle, output energy = lowest), the cell lysate was centrifuged (12,000*g,* 5 min, 4°C). The supernatants were precleared with protein A-Sepharose, and 1 μl of anti-PRMT1 rabbit polyclonal antiserum (Tang et al., 2000) was added to supernatants equalized for protein and volume (1 hr at 4°C). Protein A-Sepharose beads were washed three times with 1 ml PBS + 0.4% Triton X-100 and then with 1 ml in vitro methylation assay buffer.

In vitro methylation assays (50 μl) were performed in 80 mM Tris buffer, pH 8, containing 4 μCi L-S-adenosyl-[methyl-^{3}H]methionine (^{3}H-SAM), 2 mM PMSF, and 10 μM benzamidine at 36°C. R3 or the nucleolin-derived peptide (1.5 μg) was used to measure PRMT activity in whole-cell homogenates. The substrate for the PRMT1 immunoassay was 1.5 μg GRG peptide. Methylation of endogenous proteins in cell fractions was carried out with equal amounts of protein for 1 hr.

Gel Electrophoresis and Western Blot Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins and peptides. Fluorograms were produced as described earlier (Cimato et al., 1997). Scanning densitometry and quantitative analysis of the fluorograms were performed with software from Molecular Analyst. Detection of PRMT1 in SDS-lysates of PC12 cells was performed on PVDF (anti-PRMT1, 1:1,000). ^{125}I-labeled donkey anti-rabbit secondary antibody was used with X-ray film detection. Chemiluminescence detection of anti-ERK (Santa Cruz Biotechnology, Santa Cruz, CA) was performed using reagents supplied by Cell Signaling Technology (Beverly, MA).

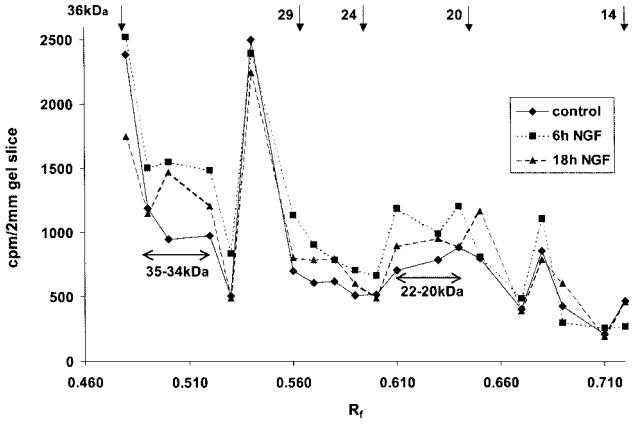

Fig. 1. NGF does not markedly affect carboxymethylation of PC12 cell proteins. PC12 cell proteins were metabolically radiolabeled by incubation with [methyl-^{3}H]methionine for 6 hr. All cells were cultured in the presence of 1% donor horse serum ± 50 ng/ml NGF. Radiolabeled proteins were separated on 7.5–15% SDS-PAGE gradient gel. The gel was fixed, stained, and dried. Carboxymethylated proteins present in contiguous 2 mm sections of the gel were detected by base hydrolysis-induced formation of ^{3}H-methanol quantified by liquid scintillation spectroscopy. The amount of radioactivity present in the gel is illustrated as a function of protein migration (R_f). These results are representative of three independent experiments. Methylproteins migrating above the 36 kDa mass marker do not exhibit consistent differences among control and 6 hr and 18 hr NGF incubation conditions. The mobility of molecular mass standards is indicated by arrows above the graph.

RESULTS

N-Methylation Is More Prevalent Than Carboxylmethylation Among the Methylated Proteins Regulated by NGF Treatment

Kujubu et al. (1993) have described differential effects of NGF and epidermal growth factor (EGF) on protein methylation in PC12 cells. Haklai et al. (1993) have also reported increases in carboxylmethylation of the 21–24 kDa proteins in NGF-treated PC12 cells. These observations in combination with our recent detection of NGF-specific increases in protein methylation (Cimato et al., 1997) led us to consider whether carboxymethylation contributes significantly to the NGF-induced increase in protein methylation. In the presence of a protein synthesis inhibitor, proteins labeled by ^{3}H-CH_3-methionine in intact cells (±NGF 6 hr and 18 hr) were separated by SDS-PAGE. Modest increases (30–50%) in methyl-esterified proteins were detected in the M_r range of ~36 kDa and between 20 and 24 kDa (Fig. 1). The work of others, cited above, detected larger changes in carboxymethylation, but the analysis focussed on membrane proteins. In the current work, total cell protein was analyzed.

Our previous studies of total cell methylproteins indicated several examples of protein methylation increased by NGF, and the magnitudes of the changes were larger (Cimato et al., 1997). Thus, to confirm the results in Figure 1, an alternative approach to detecting NGF-regulated carboxymethylated proteins utilized SDS-PAGE gels that were run in duplicate. One gel of each pair was treated with 0.2 M NaOH to hydrolyze carboxymethyl esters prior to preparation for fluorography. Although a uniform reduction in the intensities of all labeled proteins was observed, the NGF-induced increases were maintained. Analysis of the methylproteins by 2-D IEF × SDS-PAGE gels in a similar manner gives the same result. None of the NGF-induced increases is reduced by the base treatment relative to other proteins (data not shown). The stability of the radiolabeled methylprotein pattern implies that the NGF-mediated increases occur on nitrogens (R-group side chains of arginines, lysines, and histidines) rather than on the carboxyl groups of acidic amino acids.

To evaluate N-methylation on arginine residues, we performed in vitro methylation of endogenous PC12 cell proteins from subcellular fractions in the presence or absence of GAR peptides. Figure 2 illustrates a representative experiment using R3, which is known to be asymmetrically dimethylated (Najbauer et al., 1993). Addition of R3 to extracts containing ^{3}H-SAM markedly reduces the methylation of nearly all of the endogenous proteins. All NGF-regulated methylproteins are affected, with the exceptions of proteins with mobilities of ~47 and 20 kDa (Fig. 2). The intensity of the radiolabeled 47 kDa protein is slightly reduced, but it still exhibits NGF stimulation at 5 hr and 1 day. The radiolabeling of the 20 kDa protein is elevated in the presence of R3. These results indicate that the 47 kDa protein band (perhaps several proteins) is partially N-methylated on arginine residues. The methylation of the 20 kDa protein is catalyzed by a non-PRMT enzyme. Similar marked reductions in methylation are observed in cytoplasmic extracts, but NGF-induced increases are not as numerous (data not shown). Two other GAR peptides (GRG and the nucleolin-derived peptide) also reduce methylation of endogenous proteins (cf. Fig. 6). These results, in conjunction with previous findings (Cimato et al., 1997), focus attention on the effects of NGF on cellular PRMT activity.

To determine whether PRMT activity is elevated in PC12 cells, an in vitro assay was devised using whole-cell homogenates as the source of PRMT and the R3 peptide as a substrate (Fig. 3). Radiolabeling of the peptide is elevated greater than twofold at incubation times longer than 5 min. Similar results are obtained with the nucleolin-derived peptide. Incubations with peptide alone or the PC12 cell extract alone do not exhibit measurable incorporation of tritium. Several conclusions can be derived from these experiments. PRMT activity is constitutively expressed and N-methylprotein substrates are abundant in PC12 cells. Moreover, NGF stimulates PRMT activity. The increased PRMT activity is maintained after the preparation of cell-free extracts and is manifested when either endogenous proteins or exogenous GAR peptides are used to measure the activity.

NGF Activation of PRMT1

Recently, Tang et al. (2000) demonstrated that PRMT1 is the catalytic subunit of the principal PRMT in

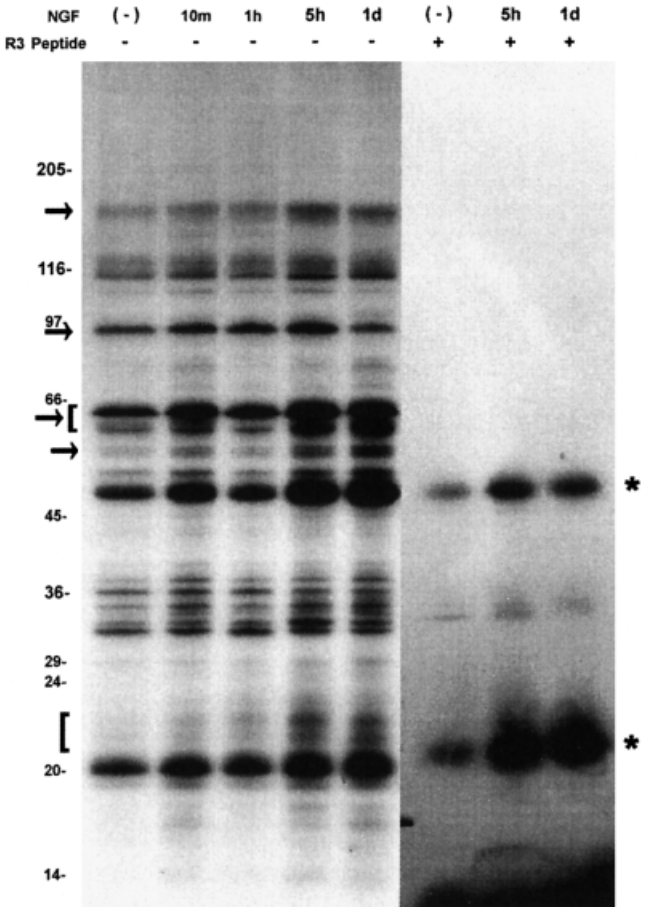

Fig. 2. GAR peptide derived from fibrillarin, R3, inhibits the N-methylation of NGF-sensitive PC12 cell substrates in vitro. PC12 cell nuclear extracts were prepared (Cimato et al., 1997) from control cells (–) and from cells exposed to 50 ng/ml NGF for 10 min to 1 day. The activity of endogenous methyltransferases was determined using 100 μg of protein (±300 μM R3 peptide). Proteins were separated on a 7.5–15% polyacrylamide gradient gel. Arrows and brackets mark several positions of increased protein methylation. Asterisks indicate two protein bands not affected by the presence of R3 peptide.

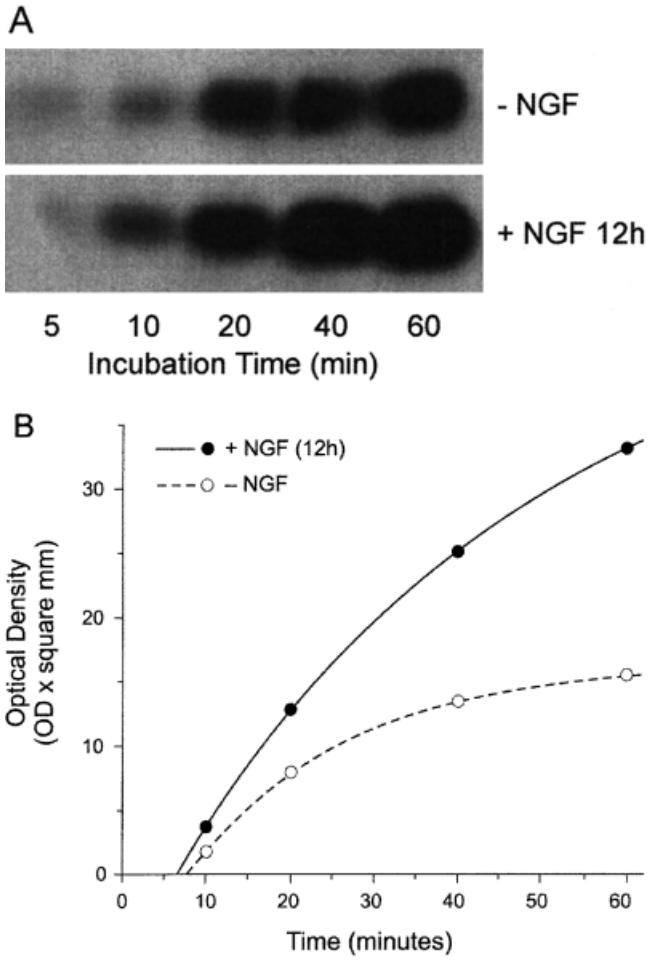

Fig. 3. NGF activates arginine methyltransferase activity in intact PC12 cells. Duplicate cultures of PC12 cells were prepared 2 days prior to treating one of the two cultures with 50 ng/ml NGF for 12 hr. **A:** In vitro assay of methyltransferase activity, as described under Materials and Methods, was performed in the presence of 1.5 μg AcGGRGGFGGRGGFGGRGGFG-NH_2 (R3) and 1 μg of PC12 cell homogenate for the durations indicated at the bottom. The R3 peptide was isolated on a 12% polyacrylamide gel. **B:** Scanning densitometry of the fluorographic image is illustrated graphically. A second independent experiment produced similar results.

mammalian cells. To determine whether PRMT1 is involved in the phenomena described above, PRMT1 antiserum was used to isolate PRMT1 from cell lysates. Figure 4A illustrates the increased methylation of the GRG peptide associated with PRMT1 immunoprecipitates from extracts of NGF-treated cells. An equivalent amount of IgG from normal rabbit serum does not immunoprecipitate measurable PRMT activity (data not shown). Figure 4B summarizes the quantitative changes in immunoprecipitable PRMT1 activity in response to NGF. Overnight treatment (16 hr) with NGF produces a 1.9-fold increase (±0.1 SD, n = 4) in activity relative to that measured in an equivalent amount of protein from non-NGF-treated cells. In long-term NGF-treated cells (>10 days), there is a 4.4-fold (±1.1 SD, n = 3) increase in enzymatic activity.

To determine whether the changes in enzyme activity are secondary to increased PRMT1 expression induced by NGF treatment, equal proteins from whole-cell lysates were evaluated by western blotting with the anti-PRMT1 antiserum. In five independent experiments, the protein immunoreactivity at 40.5 kDa (Tang et al., 1998) detected by the PRMT1 antiserum indicates no induction of PRMT1 expression. The mean OD (mm^2) from the protein band derived from NGF-treated cells was nearly identical to that from control, non-NGF-treated cells (0.96 ± 0.06 and 1.1 ± 0.21 SD for overnight NGF treatment and 14 days NGF, respectively). Figure 5A illustrates a representative result from cells treated with NGF for 14 days, a time sufficient for all cells to exhibit robust neurite out-

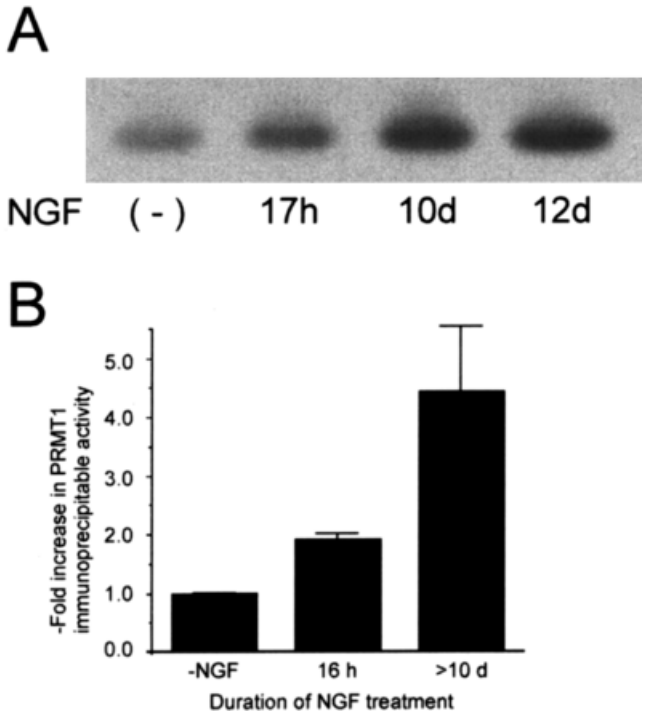

Fig. 4. NGF activates PRMT1 in intact PC12 cells. PC12 cells were stimulated with 50 ng/ml NGF for the durations indicated. **A:** Immunoprecipitation of PRMT1 was performed as described under Materials and Methods. The immunoprecipitated protein was incubated with 1.5 μg GRG in vitro for 10 min at 36°C. The radiolabeled peptide was isolated on a 12% polyacrylamide gel. **B:** The increase in PRMT1 activity is given by the ratio of optical density volumes (OD × mm^2) of the radiolabeled GRG peptide observed after in vitro methylation using extracts of NGF-treated cells relative to that of control, non-treated cells. The OD of the radiolabeled peptide incubated with immunoprecipitates from control, nontreated cells was set to be equal to 1.0 in each experiment. The graph summarizes three independent experiments. Error bars denote the standard errors of the means.

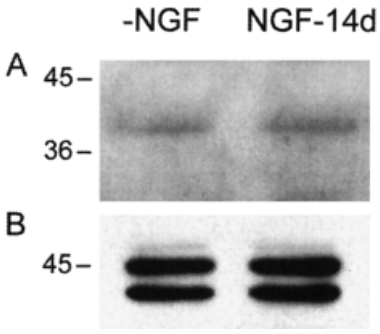

Fig. 5. Long-term NGF treatment does not affect steady-state protein levels of PRMT1 relative to total cell protein. PC12 cells were cultured in the presence (NGF-14d) or absence (–NGF) of NGF and total cell lysates prepared in SDS sample buffer for gel electrophoresis. Fifty micrograms of protein were loaded in each lane, and two equivalent western blots were probed for PRMT1 (**A**) and ERKs 1 and 2 (**B**). The PRMT1 image is derived from an ^{125}I-secondary antibody generated autoradiogram and the ERK result from chemiluminescent exposure of X-ray film.

growth. Figure 5B shows the result of a replicate western blot of the same cell lysates in Figure 5A probed for ERKs 1 and 2, the expression of which is not altered by NGF treatment. Thus, Figure 5B demonstrates that the protein loaded in each lane of the western blot in Figure 5A is equivalent. These results are in agreement with unpublished serial analysis of gene expression (SAGE) data from PC12 cells treated with NGF for 9 days. Based on this analysis (Angelastro et al., 2000), PRMT1 mRNA does not change significantly in the NGF-treated cells. Taken together, all of these studies indicate that NGF-stimulated increases in PRMT activity are due to increased catalytic activity of PRMT1, without increased PRMT1 expression.

Myelin Basic Protein, a Type II Substrate, Is Less Effective Than GAR Peptide as a Competitor of Cellular Substrates for Endogenous PRMT Activity

The results described above indicate a role for type I protein arginine methylation in NGF signaling. To determine whether type II is also involved, the capacity of type I and II substrates to act as selective competitive inhibitors of endogenous PRMTs (Najbauer et al., 1993; cf. Fig. 1) was used to distinguish proteins likely to be subtype specific.

Three different competitor substrates, added at equimolar concentrations to nuclear extracts from NGF-treated cells, incorporate tritium from ^{3}H-SAM (Fig. 6A, large arrows). Endogenous PRMTs in the extract are, thus, clearly able to methylate each of the competitors. Myelin basic protein, a type II substrate, has the least inhibitory effect on the labeling of nuclear proteins. The area under the curve (AUC) for the peak profile generated by scanning densitometry of the myelin basic protein lane was reduced by an average of 20% relative to that of the lane of extract without competitor. The nucleolin-derived peptide, a type I substrate, reduces labeling toward control, non-NGF-treated levels, by an average reduction in methylation of 62%. The greatest competitive effect is due to an artificial GAR peptide comprising seven tandem repeats of GRG. This peptide reduces in vitro methylation in the nuclear extract from NGF-treated cells to an average level equivalent to that of nuclear extract from control, non-NGF-treated cells (Fig. 6B).

Several competitor-selective effects are also observed with regard to specific protein bands. The nucleolin-derived peptide, but not myelin basic protein, inhibits methylation of proteins of M_r 116, 95, 64, 48, and 24 kDa (Fig. 6A, open arrowheads). Myelin basic protein selectively inhibits a 45 kDa protein that is not affected by the nucleolin-derived peptide (Fig. 6A, small arrow). Myelin basic protein also promotes increased methylation of a 58 kDa protein. The artificial GRG peptide is effective at inhibiting all of the NGF-regulated protein methylations, to varying degrees. All three competitors significantly reduce the methylation of nuclear proteins migrating at M_r 62, 36, 34, 32, and 30 kDa (Fig. 6A, solid arrowheads). In

summary, the nucleolin-derived peptide interferes with many of the NGF-regulated methylproteins, whereas MBP selectively inhibits at least one protein (M_r 45 kDa) that is not inhibited by the nucleolin-derived peptide. The artificial GRG peptide has the ability to inhibit the methylation of both type I and II substrates equally. These results indicate that most NGF-regulated nuclear proteins exhibit type I arginine methylation. Distinct but more modest effects of MBP suggest that type II substrates are also present.

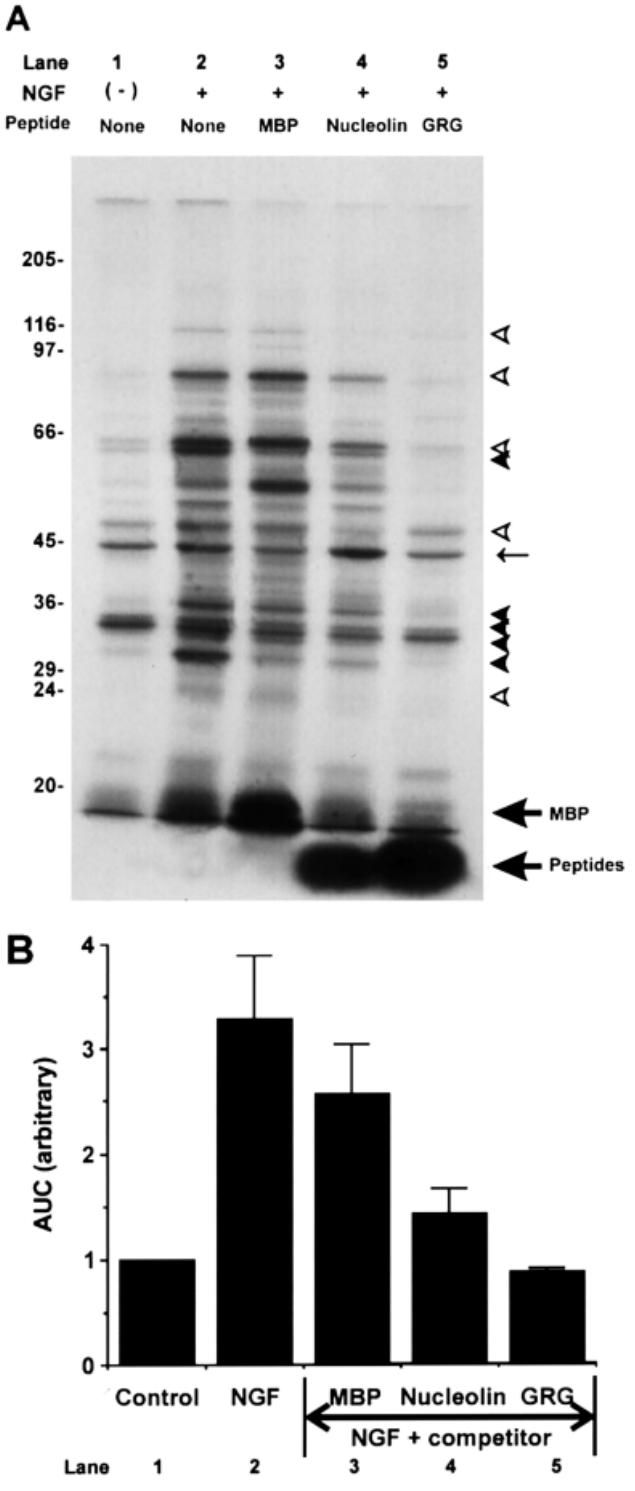

Fig. 6. GAR peptides derived from nucleolin or comprising repeating GRG effectively inhibit in vitro methylation of endogenous PC12 cell nuclear proteins, but MBP at an equimolar concentration is a poor inhibitor. Equal quantities of nuclear extract protein (100 μg) from control or NGF-treated (18 hr) cells were subjected to in vitro methylation for 60 min at 36°C. Proteins were separated on a 10% polyacrylamide gel. **A:** Nucleolin-derived peptide, GRG peptide, or myelin basic protein was present in the incubation mixture (12 μM) as indicated above the relevant lanes. The positions of the peptides and myelin basic protein are marked by the large arrows and coincide with Coomassie-stained material in the gel. Open arrowheads denote decreased labeling associated with the presence of nucleolin-derived peptide, but not MBP. Solid arrowheads indicate decreased labeling that is associated with all three competitors. Decreased labeling of a 45 kDa protein in the presence of MBP is marked by a small arrow. **B:** Estimation of total in vitro methylation by endogenous methyltransferases present in nuclear extracts was performed by scanning densitometry of each of the lanes in A from 22 kDa to 205 kDa. The average area under the curve (AUC) for the peak profiles of each condition is plotted. Error bars depict the standard errors of the means from four independent experiments.

DISCUSSION

The Predominant Methylprotein Targets of NGF Action Are Type I Substrates

The action of NGF involves a cascade of intracellular signals that mediate many responses, including differentiation, cell survival, and regulation of cell structure and gene expression. Findings from this laboratory have demonstrated that NGF regulation of protein methylation plays a role in neurite outgrowth (Cimato et al., 1997). Carboxylmethylation of Ras and other small G proteins have previously been implicated in NGF-specific signal transduction (Qiu and Green, 1991; Kujubu et al., 1993). The present findings, however, indicate that carboxylmethylation is not as prevalent among NGF-regulated methylproteins as is arginine N-methylation.

In the presence of exogenous type I PRMT substrates, the methylation of nearly all NGF-regulated methylproteins is reduced (Figs. 2, 6). These results emphasize the quantitative importance of type I PRMT substrates as targets of NGF signaling. PRMT1 is the predominant type I enzyme in RAT1 cells and mouse liver (Tang et al., 2000). Based on the current work, it is likely that PRMT1 is predominant in PC12 cells as well. The data presented here demonstrate further that NGF activates PRMT1 in a sustained manner within 24 hr after stimulation (Fig. 4). This finding represents the first example of an NGF-activated methyltransferase. The mechanism responsible for NGF activation of PRMT1 does not involve increased expression of the enzyme (Fig. 5). To learn more about the mechanism, future studies will examine NGF effects on protein methylation in cells that express TrkA mutants.

The Selectivity and Degree of the Inhibition of Protein Methylation Are Different Among Three Competitor Substrates

Myelin basic protein, a type II PRMT substrate, is methylated by an activity in nuclear extracts of PC12 cells (Fig. 6A), but NGF does not produce consistent increases in type II activity (unpublished data, Eckler, Tascione and Aletta) when myelin basic protein is used as an in vitro substrate. Myelin basic protein is also much less effective than the type I nucleolin-derived peptide for the inhibition of in vitro methylation (Fig. 6). Nevertheless, myelin basic protein does produce decreased labeling of proteins in the molecular mass range of 32–38 kDa. Inhibition of a 45 kDa methylation product is also observed with myelin basic protein, but not with the nucleolin-derived peptide. Thus, type II enzyme and substrate are present in PC12 cells. The nature of the enzyme and the potential for regulation, however, require further analysis.

The GRG peptide is the most effective competitor of the three exogenous substrates. A similar heptapeptide of repeating GRG residues has also been shown to be the most effective methyl acceptor in a substrate specificity analysis using a series of peptides and protein methylase I, the designation for biochemically purified PRMT1 from calf brain (Rawal et al., 1995). One explanation for the differential effects of the competitor substrates may be related to amino acid sequence. Although the extent of the diversity of arginine methylation sites is not presently known, some inferences based on experimental facts can be proposed. Type I arginine methylation sites in proteins have been unequivocally demonstrated in only a small number of proteins (Gary and Clarke, 1998; Smith et al., 1999; Belyanskaya et al., 2001). By sequence comparisons among all of the mapped sites of arginine dimethylation, the arginine residue is followed by glycine. The presence of another glycine N-terminal to the arginine site of methylation is also found in approximately 40% of the cases of confirmed asymmetric protein arginine methylation (Gary and Clarke, 1998). This GRG motif is also present in the recently discovered methylprotein RBP16 (Pelletier et al., 2001). It is, furthermore, interesting that GRG is the minimal motif for MBP methylation and is also found in all nine of the confirmed symmetrical dimethylarginines of Sm protein D1 and three of the four confirmed symmetrical dimethylarginines of Sm D3. Thus, the artificial GRG peptide may be the most nonselective of the three competitor substrates, because it contains the longest uninterrupted arginine methyl-acceptor motif.

The other GAR peptides used in this study contain variations of glycines and phenylalanine residues between the arginines. The GRG sequences of Sm D1 and D3 are not interrupted by phenylalanines, and the GRG of myelin basic protein occurs in an otherwise unremarkable stretch of amino acids. The additional amino acids in myelin basic protein and the nucleolin-derived and R3 peptides introduce molecular features different from the artificial, repetitive GRG peptide, which may impose unique constraints on recognition by type I or type II enzymes.

Potential Functions of Protein Arginine Methylation

Unlike protein phosphorylation, N-methylation does not appreciably change the charge on the posttranslationally modified protein. Methyl groups, particularly dimethylation at multiple sites in a GAR sequence, possibly influence protein dynamics by interfering with intermolecular interactions. This may involve other proteins or RNA or DNA. Bedford et al. (2000) recently demonstrated that the substitution of a single asymmetric dimethylarginine for an unmodified arginine within the HIV-1 Nef protein disrupts binding to $p59^{fyn}$. Methylation of the arginine residue of a fibronectin peptide (RGD) also prevents fibronectin-mediated inhibition of mitosis, presumably by hindering interactions between fibronectin and integrins (Hyun et al., 2000).

Jones et al. (2001) performed a computational analysis of the intermolecular interfaces of 32 protein–RNA complexes and found that arginine plays a key role in protein–RNA binding. In the experiments described here, the most numerous NGF-mediated increases in methylation were observed in nuclear extracts. Several hnRNPs are methylproteins involved in mRNA processing and cytoplasmic–nuclear shuttling. Recently, two novel PRMT-interacting proteins were discovered that may regulate hnRNP methylation, which may help to explain the role of hnRNP transport in gene expression during cell differentiation (Inoue et al., 2000). Finally, arginine methylation may play a role in protein–DNA interactions. Hormone receptor signal transduction that results in increased transcription involves CARM1 (Chen et al., 1999). This PRMT binds to p160 coactivators and enhances transcriptional activation by nuclear receptors when TIF2 or SRC-1 is coexpressed. All of the enhanced transcriptional activity of a reporter gene is hormone dependent. It is not presently known whether the hormone-dependent CARM1 pathway and the NGF activation of PRMT1 have common molecular features.

ACKNOWLEDGMENTS

Dr. Laurie K. Read (Department of Microbiology, UB School of Medicine) provided critical comments regarding the manuscript. The expert technical assistance of Peter J. Filocamo is greatly appreciated. This work was supported by NIH grant NS 40533 to J.M.A.

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