Analysis of Protein Arginine Methylation and Protein Arginine-Methyltransferase Activity

Kerri A. Mowen and Michael David

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The authors are in the Division of Biology and University of California at San Diego Cancer Center, University of California, San Diego, Bonner Hall 3138, 9500 Gilman Drive, La Jolla, CA 92093, USA. E-mail: mdavid@biomail.ucsd.edu

ABSTRACT

Posttranslational modification of proteins allows cells to adapt and react quickly to their environment beyond the boundaries set forth by genetic code. Arginine methylation, a protein modification discovered almost 30 years ago, has recently experienced a renewed interest as several new arginine methyltransferases have been identified and numerous proteins were found to be regulated by methylation on arginine residues. Until recently, the detection of arginine methylation required the use of chromatography and mass-spectrometrical analysis. The following protocol provides guidelines for the straightforward identification of arginine-methylated proteins, made possible by the availability of novel, commercially available reagents.

INTRODUCTION

Posttranslational modification of proteins is a hallmark of signal transduction and it allows existing proteins to react rapidly to extracellular events cascading to a total cellular response. In this context, protein phosphorylation on serine, threonine, or tyrosine residues has been extensively studied. Investigations of protein phosphorylation have been driven in part by the development of well-characterized and commercially available reagents. In particular, antibodies to phosphotyrosine and to specific phosphorylated forms of proteins have simplified the detection of phosphorylated proteins.

Although much of the effort in signal transduction research has focused on protein phosphorylation, numerous other posttranslational modifications have been identified by which a protein may be altered in response to environmental stimuli (for example, ubiquitination, glycosylation, and acetylation). Part of the challenge of investigating new modes of posttranslational modification is to develop reagents that facilitate detection of these modifications.

Recently, we characterized the protein arginine methylation of STAT1 as a signaling requirement for interferon α/β (IFNα/β)-induced transcriptional induction (*1*). STAT1 arginine methylation was catalyzed by the protein arginine methyltransferase PRMT1, and methylation of STAT1 could be inhibited by the methyltransferase inhibitor 5′-methylthioadenosine.

Two classes of protein arginine methyltransferases have been characterized and classified based on the symmetry of their reaction products (*2*). Both type I and type II enzymes generate N^G-monomethyl-arginine. However, whereas type I protein arginine methyltransferases (PRMT1, PRMT3, CARM1) account for the formation of asymmetric N^G,N^G-dimethyl-arginine, type II enzymes catalyze the formation of symmetric N^G,N^G-dimethyl-arginine (*1*).

Investigations into the biological role of arginine methylation have been complicated by the lack of specific reagents facilitating the detection of methylated arginine residues. In vitro methylation assays such as that described below only illustrate that a protein can be modified in such a manner and leave unanswered the question of physiological relevance. Thus far, however, chromatographic methods combined with mass spectrometrical analysis has been required to demonstrate that such protein arginine methylation indeed occurs in vivo (*3*). Newly available monoclonal antibodies directed against methylated arginine residues allowed us for the first time to use straightforward immunological techniques to determine the arginine methylation status of STAT1.

The current protocol describes methods for in vitro methylation reactions, as well as for the analysis of arginine methylation of proteins in vivo, using STAT1 as an example substrate.

MATERIALS

In Vitro Arginine Methylation Assays

3-L flasks

4× SDS Gel Loading Buffer

Ampicillin

Amplify solution (Amersham Pharmacia Biotech)

Bacto-tryptone

Bacto-yeast extract

Competent *E. coli* BL21 (Invitrogen, Carlsbad, CA)

Dithiothreitol (DTT)

Glutathione (Sigma Aldrich)

Glutathione Sepharose 4B Beads (GSH-beads) (Amersham Pharmacia Biotech)

Glutathione-*S*-transferase (GST)-PRMT1 plasmid

GST-STAT1(NH_2) plasmid (pGEX, Amersham Pharmacia Biotech)

Hyperfilm MP (Amersham Pharmacia Biotech)

Isopropylthio-β-D-galactopyranoside (IPTG) (Sigma Aldrich)

NAP-5 column (Amersham Pharmacia Biotech)

Phosphate-buffered saline (PBS), 1× (Irvine Scientific, Irvine, CA)

Protease Inhibitor Cocktail Set II (CalBiochem, P/N 539132)

Ready Gels (Bio-Rad)

S-adenosyl-L-methionine (AdoMet) (Amersham Pharmacia Biotech)

S-adenosyl-L-[*methyl*-^{3}H] methionine ([*methyl*-^{3}H] AdoMet) (Amersham Pharmacia Biotech)

Screw-cap conical tubes (15 ml)

In Vivo Analysis of Arginine Methylation

Cell culture reagents and chemicals

U266 cells (ATCC)

Fetal Bovine Serum (FBS) (Irvine Scientific)

L-Glutamine (Irvine Scientific)

Penicillin/Streptomycin solution, 100× (Irvine Scientific)

RPMI 1640 (Irvine Scientific)

Hepes, pH 7.9

NaCl

NaF

Triton X-100

β-glycerophosphate

Sodium orthovanadate

Phenylmethylsulfonyl fluoride (PMSF)

Antibodies and immunoprecipitation reagents

Either of two antibodies will be acceptable:

Mouse monoclonal antibody to methylarginine, clone 7E6 (IgG1) (Abcam, Cambridge, UK) (Data sheet: http://www.abcam.com/public/ab_detail.cfm?intAbID=412)

Note: According to the manufacturer's description, this antibody reacts with mono- and asymmetric dimethylated arginine residues.

or

Mouse monoclonal antibody to methylarginine, clone 21C7 (IgM) (Abcam) (Data sheet: http://www.abcam.com/public/ab_detail.cfm?intAbID=413)

Note: According to the manufacturer's description, this antibody reacts with asymmetric dimethylated arginine residues only.

Monoclonal antibody to STAT1 (BD Transduction Laboratories)

Antibodies to mouse IgM (for use with 21C7 only)

IgG1 or IgM isotype control antibodies

Protein G Sepharose (Amersham Pharmacia Biotech)

EQUIPMENT

Cell sonicator

Shaking incubator

Apparatus and appropriate reagent systems for SDS-PAGE and Western blot

RECIPES

Recipe 1: 2× YT Media

Bacto-tryptone	16 g
Bacto-yeast extract	10 g
NaCl	5 g
Water	900 ml

Adjust to pH 7.0 with 1 M NaOH. Adjust volume to 1L. Autoclave.

Recipe 2: 50 mM DTT

Prepare a 50-mM solution in H_2O and store at –20°C.

Recipe 3: GSH-Slurry

Remove a bed volume of 0.5 ml of GSH-beads and incubate with 500 µl of 50 mM DTT (Recipe 2) for 10 min at 4°C. Sediment beads by centrifugation at 2000 rpm in a microcentrifuge for 2 min and equilibrate beads 3 times with 1 ml of 1× PBS. Resuspend the beads in 0.5 ml of 1× PBS after the final equilibration to each tube. Store beads at 4°C or on ice until used.

Recipe 4: 20 mM Glutathione

Prepare a 20-mM solution in 1× PBS immediately before use. Store on ice.

Note: Glutathione will change the pH of PBS, so readjust the pH to 7.4.

Recipe 5: Gel Fix Solution

Prepare gel fix solution containing 10% (v/v) acetic acid: 10% (v/v) methanol in water.

Recipe 6: RPMI Culture Medium

Add FBS to a final concentration of 10%. Sterile filter through a 0.2-µm filter.

Recipe 7: Lysis Buffer

Hepes, pH 7.4	20 mM
Triton X-100	1%
NaCl	100 mM
NaF	50 mM
β-glycerophosphate	10 mM
Sodium orthovanadate	1 mM
PMSF	1 mM

PROCEDURES

In Vitro Arginine Methylation Assays

Purification of fusion proteins

1. To a 3-L flask containing 100 ml of 2× YT Media (Recipe 1) and 100 µg/ml of ampicillin, add one colony of BL21 *E. coli* transformed with the GST-PRMT1 plasmid. To a second flask containing the same amount of 2× YT Media (Recipe 1) and 100 µg/ml of ampicillin, add one colony of BL21 *E. coli* transformed with GST-STAT1(NH_2).
2. Grow at 37°C overnight while shaking at 300 rpm.
3. In the morning, add 900 ml of 2× YT Media (Recipe 1) with 100 µg/ml ampicillin to the overnight cultures. Incubate at

37°C for 3 hours while shaking.

4. Add IPTG to a final concentration of 0.1 mM. Incubate at 37°C for 6 hours while shaking.
5. Centrifuge the bacterial suspension at 12,000*g* rpm for 15 min at 4°C to pellet the bacteria. Discard supernatant.
6. Resuspend the pellet in 30 ml of 1× PBS containing 1 ml of Protease Inhibitor Cocktail Set II.
7. Sonicate the cells on ice for 30 s.
8. Cool the samples on ice for 5 min.
9. Repeat sonication and cooling five times.

   Note: For best results, optimize your sonicator for maximal cell fracturing according to the recommendations of the manufacturer of the sonicator.
10. Centrifuge cell lysate at 12,000g for 25 min at 4∞C.
11. Transfer supernatants into two screw-cap 15-ml tubes each.

    Note: It is important to split each sample into two 15-ml tubes instead of one 50-ml tube, in order to reduce the air volume in the tube.
12. Add 0.5 ml of GSH-Slurry per tube (Recipe 3). Incubate with agitation for 2 hours at 4°C.
13. Sediment GSH-beads in the 15-ml tubes at 1500*g* for 5 min.

    Note: Save the supernatant in case the fusion protein did not bind to the beads efficiently.
14. Wash GSH-beads two times with 10 ml of ice-cold 1× PBS. Sediment the beads at 1500*g* for 5 min each time.
15. Add 1 ml of ice-cold 1× PBS and transfer the beads to a single microcentrifuge tube.
16. Pellet the beads at 1500*g* for 5 min and discard supernatant.

    Note: This is the final step for PRMT1 purification; use the fusion protein attached to the beads for the methylation reaction. For STAT1, continue with the elution and additional purification steps before using in the methylation reactions.

Elution and additional purification for STAT1 preparation

1. Elute the STAT1 fusion protein by adding 100 µl of 20 mM glutathione (Recipe 4) to the microcentrifuge tube and agitate at 4°C for 1 hour.
2. Sediment the beads at 5000*g* for 5 min and collect the supernatant.
3. Add 200 µl of 20 mM glutathione (Recipe 4) and agitate at 4°C for 1 hour.
4. Sediment beads at 5000*g* and collect the supernatant.
5. Repeat steps 3 and 4 twice, then pool the supernatants of all four elutions.
6. Equilibrate a NAP-5 column with 10 ml of 1× PBS. Let 1× PBS pass through the column. Discard column eluate.
7. Load the combined eluates onto the bed of the column. Allow the sample to completely enter the column.
8. Add 1.0 ml of 1× PBS to the column and collect eluates in 100 µl fractions. Most of the protein should elute in fractions 5 through 8.
9. Measure the protein concentration and perform SDS-PAGE to determine the amount and purity of the protein.

Methylation reactions

These reactions are performed using [*methyl*-3H]AdoMet; therefore, the proper precautions should be taken for safe handling and disposal of radioactive materials. A control sample should also be prepared with unlabeled AdoMet to confirm the specificity of the reaction.

1. Resuspend the GST-PRMT1 beads in 1× PBS to a 50% slurry.
2. Combine 1 µg of GST-PRMT1 (as beads), 10 µg of STAT1 protein or other substrate, and 2 µl of [*methyl*-^{3}H]AdoMet. Adjust the final volume of the reaction to 80 µl with 1× PBS.

   Note: It is important to resuspend the stock of PRMT1 beads thoroughly each time before removing them, because they settle quickly to the bottom of the tube.
3. Incubate the reaction at 37°C for 90 min.

4. Stop the reaction with 20 µl of 4× SDS Gel Loading Buffer and boil for 5 min.
5. Load 25 µl on a 10% Tris-HCl Ready Gel.
6. After electrophoresis, fix the gel in gel fix solution (Recipe 5) for 20 min.
7. Remove the fixing solution.
8. Add enough Amplify solution to cover the gel and agitate the gel for 30 min.
9. Rinse the gel under running water for several minutes.
10. Dry the gel and expose to Hyperfilm MP overnight, or until a satisfactory exposure is achieved.

    Note: Make sure to dispose of radioactive solutions and reagents properly and in accordance with your institution's regulations.

In Vivo Analysis of Arginine Methylation

In the following protocol, the in vivo arginine methylation status of STAT1 is determined by immunoprecipitation with antibodies to methylarginine, separation of the immunoprecipitates by SDS-PAGE, and, following transfer, immunoblotting for STAT1. In addition, controls are suggested to ensure the specificity of the immunoprecipitations.

Immunoprecipitation with antibodies to methylarginine

The concentrations of the antibodies to methylarginine are not defined and seem to vary by lot, so the optimal amount of antibody must be empirically determined. The amount used in this protocol should be considered a starting point for titrations. It is also important to have high (at least 1 mg/ml) protein concentrations in the samples.

1. Seed approximately 3×10^6 U266 cells (enough to yield at least 1 mg of protein) in 5 ml of RPMI Culture Medium (Recipe 6) in a small dish or flask. Grow the cells in a humidified 5% CO_2 chamber at 37°C until enough cells are present for assays.

    Note: The addition of AdoMet (1 mM) to the tissue culture medium may be helpful in increasing the amount of methylated proteins.

2. Harvest the cells by centrifugation at 2000*g* for 5 min.
3. Rinse the cell pellet with ice-cold 1× PBS. Centrifuge at 2000*g* for 5 min.
4. Resuspend the pellet in 0.5 ml of Lysis Buffer (Recipe 7) and transfer to 1.5-ml microcentrifuge tubes.
5. Incubate on ice for 15 min.
6. Vortex to mix cells.
7. Centrifuge at 13,000*g* for 10 min at 4°C.
8. Incubate lysate with 20 µl of 50% Protein G Sepharose suspension for 1 hour at 4°C to decrease nonspecific binding.
9. Centrifuge the beads at 13,000*g* for 1 min at 4°C.
10. Transfer the supernatants to new microcentrifuge tubes.
11. Add 15 µl of antibodies to methylarginine (7E6 or 21C7) and 30 µl of 50% 1× PBS-Protein G Sepharose suspension. Control samples should get 15 µl of IgG1 or IgM isotype control antibodies and 30 µl of 50% 1× PBS-Protein G Sepharose suspension.

    Note: Add antibodies to mouse IgM to the samples if 21C7 is used for the immunoprecipitation because the IgM antibodies will not bind efficiently to the Protein G Sepharose beads.

12. Incubate overnight at 4°C.
13. Sediment the beads at 13,000*g* for 1 min at 4°C.
14. Remove the supernatant and wash beads three to five times with cold Lysis Buffer (Recipe 7), centrifuging at 13,000*g* for 1 min at 4°C after each wash.
15. After the final wash, remove the supernatant and add 40 µl of 4× SDS Gel Loading Buffer to the beads and perform a Western blot to detect STAT1.

    Note: Although both of the antibodies to methylarginine immunoprecipitated reasonably well, neither one of them worked for Western blotting in our hands. Thus, the methylated protein must be detected with an antibody to the protein of interest.

NOTES AND REMARKS

Controls

Recombinant proteins [such as GST-STAT1(NH_2)] that have been methylated in vitro using nonradiolabeled AdoMet as described in the previous protocol can serve as positive controls for the effectiveness of the immunoprecipitation.

Preincubation of the antibodies to methylarginine with unmethylated or in vitro methylated recombinant proteins as competitors can serve as an additional control for the specificity of the immunoprecipitation.

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