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Detection of Post-translational Modifications of Replication Protein A

Cathy S. Hass, Ran Chen, and Marc S. Wold

Department of Biochemistry, Carver College of Medicine, University of Iowa 375 Newton Rd.,
3107 MERF, Iowa City, IA 52242, USA

Marc S. Wold: marc-wold@uiowa.edu

Summary

Replication Protein A (RPA) is a single-strand DNA-binding protein that is found in all eukaryotes. RPA is subjected to multiple post-translational modifications including serine- and threonine-phosphorylation, poly-ADP ribosylation and SUMOylation. These modifications are believed to regulate RPA activity through modulating interactions with DNA and partner proteins. This article describes two methods used to detect post-translational modified RPA: immunofluorescence and immunoblotting.

Keywords

DNA replication; DNA Repair; Recombination; Immunoblotting; Immunofluorescence; Protein phosphorylation

1. Introduction

Replication protein A (RPA) is a conserved single-stranded DNA-binding protein that is found in all eukaryotic cells (1, 2). RPA binds ssDNA intermediates in DNA replication, DNA repair, recombination and is involved in damage recognition in the cellular response to DNA damage. RPA is a stable complex composed of three subunits of approximately 70-, 32- and 14-kDa (referred to as RPA1, RPA2 and RPA3, respectively). RPA is subjected to multiple post-translational modifications. These include phosphorylation of serine and threonine residues, poly-ADP ribosylation and SUMOylation (1, 3, 4). Although, limited phosphorylation of RPA is observed in S and M phases, post-translational modification of RPA primarily occur after DNA damage (4, 5). These modifications have been mapped to both the RPA1 and RPA2 subunits. The N-terminus of RPA2 and the C-terminal domain of RPA1 are hyperphosphorylated and SUMOylated (respectively) after cells are exposed to DNA damage (4, 5). The role(s) of post-translational modifications of RPA are not well understood but are thought to modulate RPA activity and help coordinate the cellular DNA damage response (5–7).

Immunofluorescence microscopy and immunoblotting are two powerful techniques for identifying post-translationally modified proteins. Antibodies to specific post-translational modifications can be used to detect modified proteins. There are also a number of antibodies that interact with specific phosphorylated residues in RPA that are commercially available

(8, 9). This allows phosphorylation of specific sites on RPA2 to be monitored either by immunoblotting or immunofluorescence. The methods for detecting these modifications of RPA will be described below.

1.1. Detection of Post-translationally modified RPA by immunofluorescence

Immunofluorescence can be used to examine the localization of proteins throughout the cell and to determine localization of specific forms of proteins in response to different cell conditions. RPA is normally localized in the nucleus of cells and shows diffuse nuclear staining by immunofluorescence (10). When cells are exposed to DNA damage, RPA localizes to sites of DNA damage resulting in a punctate staining pattern (11, 12). This localized RPA is tightly associated with chromatin and can be visualized by immunofluorescence after detergent extraction (13). RPA is also phosphorylated and SUMOylated after DNA damage. Phospho-residue specific antibodies to RPA can be used to visualize chromatin-associated modified RPA.

1.2. Detection of post-translationally modified RPA by immunoblotting

The mobility of RPA2 and RPA1 is reduced when phosphorylated or SUMOylated, respectively. Antibodies to native RPA can be used to detect these changes in mobility after separation by SDS-PAGE using immunoblotting. Depending on the number of phosphorylated residues, the change in mobility observed after phosphorylation of RPA2 can be small. To obtain optimal separation of the different phosphorylated forms of RPA, it is necessary to separate RPA on high percentage (12–13%) polyacrylamide gels (14, 15). If it is desired to analyze multiple subunits of RPA simultaneously, the size distribution of RPA subunits (70-, 32-, and 14-kDa) calls for full size, gradient gels. Gradient gels can be purchased commercially or poured in house. The protocol below includes a description for pouring gradient SDS-PAGE gels. Phosphorylated RPA can also be detected by immunoblotting with specific anti-phospho-residue RPA2 antibody.

2. Materials

All the buffers and solutions are made in ultrapure deionized water. Buffers for immunofluorescence staining are stored at 4°C unless otherwise noted. Procedures are carried out at room temperature unless otherwise noted.

2.1. Cell culture components

1. 6 well cell culture polystyrene plates
2. 22×22 Cover glass
3. Dulbecco's Modified Eagle Medium (DMEM) plus 10% Calf Serum
4. HeLa cells
5. DNA damaging agent: camptothecin, dissolved at 14.4 mM in DMSO to make stock solution.

2.2. Immunofluorescence staining components

1. CSK buffer: 10 mM HEPES (diluted from 1 M stock at pH 7.8), 300 mM sucrose, 100 mM NaCl, and 3 mM MgCl₂ in sterile water. To make 1 liter of CSK buffer, weigh 102.7 g of sucrose, 5.84 g of NaCl, and 0.61 g of MgCl₂ and add to 800 mL of water. Add 10 mL of 1M stock of HEPES. Bring volume to 1 L with water.
2. 0.5% TritonX-100 CSK: 0.5% TritonX-100, 10 mM HEPES, 300 mM sucrose, 100 mM NaCl, and 3 mM MgCl₂ in sterile water. To make 1 liter of 0.5% TritonX-100 CSK, add 5 mL TritonX-100 to 1 L of CSK buffer.
3. Fixing solution: 10% Neutral Buffered Formalin
4. Phosphate Buffered Saline (PBS) buffer: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄. To make 1 liter of PBS buffer, weigh 8 g NaCl, 0.2 g KCl, 0.062 g Na₂HPO₄·7H₂O, and 0.168 g KH₂PO₄ to 800 mL of water. Bring volume to 1 L with water.
5. 0.5% NP-40/PBS: 0.5% NP-40 in PBS. To make 500 mL of 0.5% NP-40/PBS, add 2.5 mL NP-40 to 500 mL PBS.
6. Blocking solution: 5% calf serum in PBS. To make 500 mL blocking solution, add 25 mL calf serum to 500 mL PBS.
7. Antibody dilution buffer: 0.1% TritonX-100 in PBS. To make 100 mL antibody dilution buffer, add 0.1 mL TritonX-100 to 100 mL PBS.
8. Primary antibody: Phospho RPA32 (S33) Antibody (Bethyl Laboratories, Inc., Montgomery, TX); RPA2 antibody (71-9A) (16)
9. Secondary antibody: FITC-conjugated donkey anti-rabbit (Jackson ImmunoResearch), goat anti-mouse Texas Red (Invitrogen)
10. DAPI: 1 µg/µL 4',6-diamidino-2-phenylindole
11. Precleaned Micro Slides 1×3×1.0 mm
12. Mounting media: ProLong Antifade Kit (Invitrogen)
13. Fluorescence microscope

2.3. SDS Polyacrylamide Gel components

1. 4X Upper Tris (Stacking Buffer): 0.5 M Tris, pH 6.8, 0.4% SDS. To make 1 liter of 4X Upper Tris, weigh 78.8 g of Tris Base and 4 g of SDS, and add them to 800 ml of water. Add water to bring volume to 900 ml. Mix and adjust pH with HCl. Bring volume to 1 L with water. Store at room temperature.
2. 4X Lower Tris (Resolving Buffer): 1.5 M Tris pH 8.8, 0.4% SDS. To make 1 liter of 4X Lower Tris, weigh 236.4 g of Tris Base and 4 g of SDS, and add them to 800 ml of water. Add water to bring volume to 900 ml. Mix and adjust pH with HCl. Bring volume to 1 L with water. Store at room temperature.
3. 50% glycerol: To make 100 ml of solution, mix 50 ml of glycerol with 50 ml of water.

4. 40% Acrylamide/Bis Solution, 19:1 (Bio-Rad). Store at 4 °C.
5. 10% Ammonium persulfate (10% APS): Add 0.1 g of ammonium persulfate to 800 µl of water. Add water to bring volume to 1 ml. Make fresh 10% APS every three to four days. Store 10% APS at 4°.
6. TEMED. Store at 4 °C.
7. Water-saturated N-butanol. To make the water-saturated n-butanol, mix equal volume of water and n-butanol in a container and vortex vigorously. The top layer will be water-saturated n-butanol and the bottom layer will be n-butanol-saturated water
8. Precision plus protein standards
9. 1X SDS-PAGE running buffer: 0.1% SDS, 0.025 M Tris Base, 0.192M Glycine. To make 1 liter of 10X SDS-PAGE running buffer stock (1% SDS, 0.25 M Tris Base, 1.92 M Glycine), weigh 30 g Tris Base, 144 g glycine, 10 g SDS, mix first with 600 ml of water and bring the volume to 1 L with water. No pH adjustment needed. To make 1 L of 1X SDS-PAGE running buffer, mix 100 ml of 10X stock with 900 ml of water.
10. 30 mL gradient maker
11. Full sized SDS-PAGE gel apparatus. Protocol describes making gels for Hoefer SE600 series gel apparatus but can be adjusted to any commercial gel apparatus.

2.4. Immunoblotting components

1. 3X SDS sample buffer (0.188 M Tris-HCl, pH 6.8 at 25 °C, 6% SDS, 30% Glycerol, 2.14 M β-mercaptoethanol, 0.03% bromophenol blue). To make 10 ml of 3X SDS sample buffer, mix 3.75 ml 4X Upper Tris, 0.6 g SDS, 3.75 ml of 80% Glycerol, 1.5 ml of β-mercaptoethanol and 300 µl of 1% bromophenol blue.
2. Transfer Buffer: 25 mM Tris Base, 192 mM Glycine, 0.04% SDS, 20% Methanol. To make 1 L of transfer buffer, weigh 3 g of Tris Base, 14.4 g of Glycine, 0.4 g of SDS, mix with 200 ml of methanol and 600 ml of water. Add water to bring volume to 1 L. Store at room temperature.
3. 10X Tris Buffered Saline (TBS): 1.5 M NaCl, 0.2 M Tris Base, pH 7.6. To prepare for 1 L of 10X TBS, weigh 24.2 g Tris Base, 80 g NaCl, and add them to 800 ml of water. Adjust the pH to 7.6 with HCl. Bring the volume to 1 L with water. Store at room temperature.
4. Wash buffer (TBS/T): 1X TBS, 0.1% Tween-20. To make 1 L of TBS/T, mix 1 ml of Tween-20 with 1 liter of 1X TBS. Store at room temperature.
5. Blocking buffer: 1X TBS, 0.1% Tween-20 with 5% non-fat dry milk powder. To make 150 ml blocking buffer, add 15 ml 10X TBS to 135 ml of water and mix well. Add 7.5 g non-fat dry milk powder and 1.5 ml Tween-20 while stirring.
6. Electroblothing apparatus. Procedure described for Semi Dry Electroblothing system (Thermo scientific)

7. 3MW Blotting paper, 0.33 mm (MDSCI)
8. Nitrocellulose membranes, 0.45 μ M (Bio-Rad)
9. SuperSignal West Pico Chemiluminescent Substrate (Thermo)
10. Plastic wrap
11. X-ray film
12. Primary antibody: Phospho RPA32 (S33) antibody (Bethyl Laboratories, Inc., Montgomery, TX); RPA2 antibody (71-9A) (16)
13. Secondary antibody: HRP-conjugated anti-mouse or HRP-conjugated anti-rabbit antibody (cell signaling).

3. Methods

3.1. Cell culture

1. Sterilize coverslips in 70% ethanol in six-well tissue culture plates under UV light in tissue culture hood for 5 minutes.
2. Wash coverslips and wells with sterile PBS, twice. Leave coverslips on bottom of wells.
3. Seed wells with 5×10^5 cells. Determine number of cells per mL using a hemocytometer. Dilute with growth media to obtain 2.5×10^5 cells/mL. Add 2 mL of diluted cells per well.
4. Grow cells on coverslips in six-well tissue culture plates in DMEM with 10% calf serum at 37°C with 5% CO₂ for 24 hrs prior to starting experiments.
5. Treat cells as desired for experiment. This can involve transfecting siRNA or expression plasmids and/or treatment with drugs or DNA damaging agents. In the example shown in Figure 1, DNA damage was induced by treatment with 20 μ M camptothecin for 4 hours. Stock solution of camptothecin in DMSO was diluted 1:720 into cell growth media to obtain 20 μ M concentration. Then 2 mL of the camptothecin containing media was added to each well.

3.2. Immunofluorescence staining

- 1 Wash coverslips with 1 mL per well of CSK buffer for 2 minutes, twice.
- 2 Extract non-chromatin bound RPA with 1 mL per well of 0.5% TritonX-100 CSK for 5 minutes (see Immunofluorescence note 1).
- 3 Fix cells with 1 mL per well 10% Neutral Buffered Formalin for 20–30 minutes.
- 4 Wash coverslips with 1 mL per well PBS for 2 minutes, twice.
- 5 Treat cells with 1 mL per well 0.5% NP-40/PBS for 5 minutes for second extraction treatment (see Immunofluorescence note 1).
- 5 Wash coverslips with 1 mL per well PBS for 2 minutes, twice.

- 6 Block with 1 mL per well of 5% serum in PBS for 30–60 minutes (see Immunofluorescence note 2).
- 7 Incubate coverslips in 1 mL per well of primary antibody diluted in 0.1% Triton PBS overnight at 4°C (see Immunofluorescence note 3).
- 8 Wash coverslips with 1 mL per well PBS for 2 minutes, twice.
- 9 Incubate coverslips in 1 mL per well of secondary antibody diluted in 0.1% Triton PBS for 1–2 hours at room temperature (see Immunofluorescence note 3).
- 10 Wash coverslips with 1 mL per well PBS for 2 minutes, twice.
- 11 Incubate coverslips in 1 mL per well of DAPI diluted to 1 µg/µl in PBS for 5 minutes.
- 12 Wash coverslips with 1 mL per well PBS for 2 minutes, twice.
- 13 Mount coverslips onto slides. Remove coverslips from wells with tweezers and place cell-side up on edge of glass slide. Add approx 30 µl of mounting media (Invitrogen ProLong Antifade Kit) on each coverslip. Invert coverslip (cell-side down) on the center of the glass slide. Seal edges with clear nail polish.
- 14 Collect images on fluorescence or confocal microscope. Figure 1 shows an example of HeLa nuclei that were stained with antibodies to native RPA2 and phosphoserine 33 in RPA2. Images collected on a confocal microscope.

3.3 Pouring 8–14% gradient gel

1. Clean and assemble gel plates in pouring stand as recommended by manufacturer.
2. Set up dry gradient maker on a magnetic stir plate with tubing leading to top of assembled gel apparatus. (Make sure the gradient maker sits above assembled gel plates.) The gradient maker has two chambers for two different concentration acrylamide solutions. The high concentration will be in the chamber (with outlet) and should have a small stir bar in it.
3. The following recipe is for a 1 mm thick 18 X 16 cm gel. Volumes can be adjusted proportionally for different size or thickness gels. Make 8% and 14% acrylamide solutions for running gel. There is no need to degas solutions. The amount of TEMED and APS should cause the gel to polymerize in one hour. See Gradient Gel Note 1.

	H2O	4X Lower Tris	50% glycerol	40% Acril/Bis		TEMED	10% APS
8%	5.67 mL	2.7 mL	0.21 mL	2.18 mL	Mix	6.7 µl	13.3 µl
14%	3.25 mL	2.7 mL	1 mL	3.75 mL	Mix	6.7 µl	13.3 µl

4. Make sure the valves are in the closed position. Pour 8% acrylamide solution into the chamber of the gradient maker with a single valve. Briefly open and close valve between chambers to fill the valve with 8% solution and to make sure valve is not

blocked with air bubbles. Pour 14% acrylamide solution into the chamber with the outlet to the gel apparatus. Start stir bar and open valve at outlet to start flow into gel apparatus. If solution does not start flowing immediately, provide some positive pressure by gently pressing thumb over top chamber or by gentle suction with a syringe attached to the other end of the tubing. Once the 14% acrylamide solution is flowing through the tubing, open valve between chambers. Adjust the rate of stirring so that there is good mixing but little aeration. Schlieren lines should be observed from mixing the 8% solution with the 14% solution. Let the gradient pour until the surface is around 0.5 cm from the maximum depth of the comb or until the gradient is finished. Immediately rinse out the gradient maker with water. Then cover the gel with water saturated n-butanol. Let the gel polymerize. This should take around one hour and is indicated by the appearance of a second interface below the butanol/aqueous boundary.

5. Pick up the gel sandwich and pour off the butanol and unpolymerized acrylamide. Rinse the gel several times (>3) with water to remove any residual butanol.
6. Make 5% stacking gel. The amount of TEMED and APS should cause the stacking gel to polymerize in 15 minutes. Pipette stacking gel solution on top of running gel and insert comb.

	H2O	4X Upper Tris	50% glycerol	40% Acril/Bis		TEMED	10% APS
5%	4.2 mL	1.7 mL	0 mL	0.8 mL	mix	13.3 ul	27 ul

7. After stacking gel is polymerized, gently remove comb and rinse out wells. Rinse at least two times with water and at least one time with 1X SDS-PAGE running buffer. Shake all liquid out after each wash. Fill wells with 1X SDS-PAGE running buffer in preparation for loading samples.

3.4. Sample preparation and electrophoresis

1. Mix protein samples with 3X SDS sample buffer to make the gel samples. For 1 mm gel, samples should be 15–25 μ L with 2/3 final volume of combined water and protein sample and 1/3 volume of the 3X SDS sample buffer. Samples should contain 1–3 μ g of purified protein or 20–30 μ g of cell lysate/extracts.
2. Heat gel samples at 90°C (or boil) for two minutes.
3. Spin the tubes momentarily to get entire volume into bottom of the tube.
4. Apply the protein standards and samples to the bottoms of the wells through the buffer using gel loading tips.
5. Place loaded gel in gel running apparatus and fill both buffer chambers with 1X SDS-PAGE running buffer. For Hoefer SE600 series gel apparatus, the lower chamber holds 3–3.5 L and upper chamber holds 500 mL 1X SDS-PAGE buffer. Make sure no air bubbles are trapped under the gel. Buffer can be used for up to four gels before replacing.

6. Attach to power supply and apply current. Negative electrode is attached to top of gel. For optimal separation, run the gel at constant power at 30–40 Watts for 1–1.5 hr. At this power, gel will get very warm and must be submerged in stirring 1X SDS-PAGE buffer to keep thermal stress constant. Alternatively, gel can be separated overnight under constant voltage at 55 volts. Tracking dye should be run to bottom of gel.

3.5 Immunoblotting Electrotransfer

Either wet or semi-dry transfers can be used. Follow manufacturer's recommended protocol for set up and transfer. The following procedure is for a semi-dry electroblotting apparatus (Thermo Scientific).

1. Remove gel from apparatus. Separate gel plates with plastic spatula. Prepare gel for transfer by cutting off stacking gel with a razor blade. Running gel is then transferred to electroblotting apparatus. It is easiest to do this in two steps. First peel gel off plate by holding the plate upside down over a tray containing water (to prevent gel from sticking to tray). Gently peel one corner of the gel off the glass plate and let gravity pull gel into tray. The gel can then be transferred to the blotting apparatus when ready (step 3). Alternatively, the gel can be lifted carefully from bottom (14% section) and placed directly on blotting paper.
2. Cut the nitrocellulose membrane to the size of the gel and soak in water. Also cut 6 pieces of 20 cm X 20 cm blotting paper. (Blotting paper should just cover surface of electroblotting apparatus.) Soak blotting paper in the transfer buffer.
3. Arrange a gel-membrane sandwich by laying three layers of presoaked blotting paper on the bottom surface of the semi-dry apparatus. Place the gel on top of blotting papers, followed by the presoaked nitrocellulose membrane, and another three layers of presoaked blotting papers on the top (See Immunoblotting note 1).
4. Put the lid over the semi-dry plate, finger-tighten three knobs on the lid and connect to a high current power supply. The orientation of the sandwich should be: negative electrode-gel-nitrocellulose-positive electrode
5. Run the transfer at 500 mA and 20 V for one hour. Transfer of prestained protein standards to the membrane indicates successful transfer.

3.6 Immunoblotting

1. After transfer, wash the nitrocellulose membrane three times for 5 minutes each with TBS/T at room temperature with constant rocking.
2. Block the nitrocellulose membrane with blocking buffer for one hour at room temperature with constant rocking.
3. Wash the nitrocellulose membrane three times for 5 minutes each with TBS/T with constant rocking

4. Incubate the nitrocellulose membrane in the blocking buffer with diluted primary antibody (1:1000 for antibodies shown in Figure 2). The incubation occurs at 4°C overnight with gentle rocking (See Immunoblotting note 2).
5. Wash the membrane three times for 5 minutes each with TBS/T with gentle rocking
6. Incubate membrane with diluted HRP-conjugated secondary antibody (anti-mouse IgG HRP was diluted 1:20,000, anti-rabbit IgG HRP was diluted in 1:15,000) in TBS/T with gentle rocking for 1–2 hours at room temperature.
7. Wash the membrane three times for 5 minutes each with TBS/T with gentle rocking
8. Incubate the membrane with 1:1 mixture of luminol and peroxide solutions from Chemiluminescent Substrate kit for five minutes at room temperature. Mixture should be prepared right before using.
9. Drain the membrane by tapping one corner of membrane on tissue paper to remove excess developing solution (do not let dry), wrap the membrane in plastic wrap and expose to X-ray film (See Immunoblotting note 3.)

4. Notes

4.1 Immunofluorescence

1. Extraction steps should not be done if total RPA is to be visualized.
2. Optimal blocking solution will vary depending on the source of primary and secondary antibodies. We find that bovine serum generally is effective. However, background can usually be reduced by blocking with serum from the animal that is the source of the secondary antibody.
3. Optimal dilution will need to be determined for each antibody empirically. Usual ranges are 1:100 to 1:1000 for commercial primary antibodies and higher dilutions for more concentrated antibody stocks. Secondary antibodies are usually used at dilutions of at least 1:1000. Can use any antibody specific for RPA or a modified residue.

4.2 Gradient Gel

1. ACRYLAMIDE IS A NEUROTOXIN. WEAR PERSONAL PROTECTIVE EQUIPMENT

4.3 Immunoblotting

1. Make sure there are no air bubbles between gel and nitrocellulose membrane. These will prevent protein transfer.
2. Optimal dilution will need to be determined for each antibody empirically. Usual ranges are 1:1000 to 1:10,000 for most commercial primary antibodies and higher dilutions for more concentrated antibody stocks. Secondary antibodies are usually used at dilutions of 1:1000 to 1:20,000. Any antibody specific for RPA or a modified residue can be used.

3. An initial one-minute exposure should indicate the proper exposure time.

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Figure 1. Immunofluorescence of extracted HeLa cells

HeLa cells grown on coverslips were exposed, where indicated, to 20 μ M DNA damaging agent, camptothecin, an inhibitor of Topoisomerase II, for 4 hours to cause single-strand and double-strand breaks throughout the nucleus. Cells were then fixed and extracted to leave only chromatin associated proteins. Nuclei were then stained for DNA (DAPI), treated with mouse RPA2 antibody (RPA2; 1:1000) or anti-phospho RPA32 (S33) antibody (P-RPA2; 1:1000). The antibodies were detected with secondary antibodies and visualized using confocal microscopy.

Figure 2. Immunoblot of phosphorylated RPA

Protein samples containing either 75 μ g HeLa cell extract protein or 300 ng purified RPA or both were incubated under conditions that allow efficient hyperphosphorylation of RPA (14). Identical reactions were separated on an 8–14% gradient gel and transferred to nitrocellulose. Membrane was then incubated with a 1:15,000 dilution mouse anti-RPA2 antibody (left panel) or a 1:10,000 dilution of anti-phospho RPA32 (S33) antibody (right panel). Positions of molecular mass markers are indicated on either side of each panel. Phosphorylated forms of RPA2 (lower mobility bands) were only observed in the complete reaction (third lane). Note that the RPA2 antibody detects all forms of RPA2 including multiple phosphorylated forms while the phospho-serine33 specific antibody only detects a subset of low-mobility, phosphorylated forms. (Unphosphorylated endogenous RPA2 is observed in the extract only lanes.)