Video Article

Rab10 Phosphorylation Detection by LRRK2 Activity Using SDS-PAGE with a Phosphate-binding Tag

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Abstract

Mutations in leucine-rich repeat kinase 2 (LRRK2) have been shown to be linked with familial Parkinson's disease (FPD). Since abnormal activation of the kinase activity of LRRK2 has been implicated in the pathogenesis of PD, it is essential to establish a method to evaluate the physiological levels of the kinase activity of LRRK2. Recent studies revealed that LRRK2 phosphorylates members of the Rab GTPase family, including Rab10, under physiological conditions. Although the phosphorylation of endogenous Rab10 by LRRK2 in cultured cells could be detected by mass spectrometry, it has been difficult to detect it by immunoblotting due to the poor sensitivity of currently available phosphorylation-specific antibodies for Rab10. Here, we describe a simple method of detecting the endogenous levels of Rab10 phosphorylation by LRRK2 based on immunoblotting utilizing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) combined with a phosphate-binding tag (P-tag), which is *N*-(5-(2-aminoethylcarbamoyl)pyridin-2-ylmetyl)-*N*,*N'*,*N'*-tris(pyridin-2-yl-methyl)-1,3-diaminopropan-2-ol. The present protocol not only provides an example of the methodology utilizing the P-tag but also enables the assessment of how mutations as well as inhibitor treatment/administration or any other factors alter the downstream signaling of LRRK2 in cells and tissues.

Video Link

The video component of this article can be found at https://www.jove.com/video/56688/

Introduction

PD is one of the most common neurodegenerative diseases, predominantly affecting dopaminergic neurons in the midbrain, resulting in dysfunction of the motor systems in elderly people[1]. While most patients develop PD in a sporadic manner, there are families inheriting the disease. Mutations in several genes have been found to be linked with FPD[2]. One of the causative genes for FPD is LRRK2, in which eight missense mutations (N1437H, R1441C/G/H/S, Y1699C, G2019S, and I2020T) linked to a dominantly inherited FPD called PARK8 have so far been reported[3,4,5]. Several genome-wide association studies (GWAS) of sporadic PD patients have also identified genomic variations at the LRRK2 locus as a risk factor for PD, suggesting that abnormality in the function of LRRK2 is a common cause of neurodegeneration in both sporadic and PARK8 FPD[6,7,8].

LRRK2 is a large protein (2,527 amino acids) consisting of a leucine-rich repeat domain, a GTP-binding Ras of complex proteins (ROC) domain, a C-terminal of ROC (COR) domain, a serine/threonine protein kinase domain, and a WD40 repeat domain[9]. The eight FPD mutations locate in these functional domains; N1437H and R1441C/G/H/S in the ROC domain, Y1699C in the COR domain, G2019S and I2020T in the kinase domain. Since G2019S mutation, which is the most frequently found mutation in PD patients[10,11,12], increases the kinase activity of LRRK2 by 2 - 3 fold *in vitro*[13], it is hypothesized that the abnormal increase in phosphorylation of LRRK2 substrate(s) is toxic to neurons. However, it has been impossible to study whether the phosphorylation of physiologically relevant LRRK2 substrates is altered in familial/sporadic PD patients due to the lack of methods evaluating it in patient derived samples.

Protein phosphorylation is generally detected by immunoblotting or enzyme-linked immunosorbent assay (ELISA) using antibodies specifically recognizing the phosphorylated state of proteins or by mass spectrometric analysis. However, the former strategy sometimes cannot be applied because of the difficulties in creating phosphorylation-specific antibodies. Metabolic labeling of cells with radioactive phosphate is another option to examine physiological levels of phosphorylation when phosphorylation-specific antibodies are not readily available. However, it requires a large amount of radioactive materials and therefore involves some specialized equipment for radioprotection[14]. Mass spectrometric analysis is more sensitive compared to these immunochemical methods and became popular in analyzing protein phosphorylation. However, the sample preparation is time-consuming, and expensive instruments are required for the analysis.

A subset of the Rab GTPase family including Rab10 and Rab8 was recently reported as direct physiological substrates for LRRK2 based on the result of a large-scale phosphoproteomic analysis[15]. We then demonstrated that Rab10 phosphorylation was increased by FPD mutations in mouse embryonic fibroblasts and in the lungs of knockin mice[16]. In this report, we chose to employ a sodium dodecyl sulfate polyacrylamide

gel electrophoresis (SDS-PAGE)-based method in which a P-tag molecule is co-polymerized into SDS-PAGE gels (P-tag SDS-PAGE) for detecting the endogenous levels of Rab10 phosphorylation, because a highly sensitive antibody specific for phosphorylated Rab10 was still lacking. We have failed to detect the phosphorylation of endogenous Rab8 due to the poor selectivity of currently available antibodies for total Rab8. Therefore, we decided to focus on the Rab10 phosphorylation. LRRK2 phosphorylates Rab10 at Thr73 locating at the middle of the highly conserved "switch II" region. High conservation of the phosphorylation sites among Rab proteins might be one of the reasons why phosphospecific antibodies recognizing distinct Rab proteins are difficult to make.

The phosphorylation of Rab8A by LRRK2 inhibits the binding of Rabin8, a guanine nucleotide exchange factor (GEF) which activates Rab8A by exchanging the bound GDP with GTP[15]. Phosphorylation of Rab10 and Rab8A by LRRK2 also inhibits the binding of GDP-dissociation inhibitors (GDIs), which are essential to the activation of Rab proteins by extracting GDP-bound Rab proteins from membranes[15]. Collectively, it is hypothesized that the phosphorylation of Rab proteins by LRRK2 prevents them from activation although the precise molecular mechanism and physiological consequences of the phosphorylation remain unclear.

P-tag SDS-PAGE was invented by Kinoshita *et al.* in 2006: In this method, acrylamide was covalently coupled with P-tag, a molecule capturing phosphates with high affinity, which copolymerized into SDS-PAGE gels[17]. Because the P-tag molecules in a SDS-PAGE gel selectively retard electrophoretic mobility of phosphorylated proteins, P-tag SDS-PAGE can separate phosphorylated proteins from non-phosphorylated ones (**Figure 1**). If the protein-of-interest is phosphorylated on multiple residues, a ladder of bands corresponding to differentially phosphorylated forms will be observed. In the case of Rab10, we observe only one shifted band, indicating that Rab10 is phosphorylated only at Thr73. The major advantage of P-tag SDS-PAGE over immunoblotting with phosphorylation-specific antibodies is that phosphorylated Rab10 can be detected by immunoblotting with non-phosphorylation-specific antibodies (*i.e.*, recognizing total Rab10) after being transferred on membranes, which is generally more specific, sensitive, and available from commercial/academic sources. Another advantage of using P-tag SDS-PAGE is that one can obtain approximate estimation of the stoichiometry of phosphorylation, which is impossible by immunoblotting with phosphorylation-specific antibodies or by metabolic labeling of cells with radioactive phosphates.

Apart from the use of inexpensive P-tag acrylamide and some minor modifications related to it, the present method for detection of Rab10 phosphorylation by LRRK2 follows a general protocol of immunoblotting. Therefore, it should be straightforward and easily executable in any laboratories where immunoblotting is a usual practice, with any types of samples including purified proteins, cell lysates, and tissue homogenates.

Protocol

1. Sample Preparation for the P-tag SDS–PAGE

1. Remove and discard the media from 10 cm dishes in which cells are grown using a suction and wash cells with 5 mL Dulbecco's phosphate-buffered saline (DPBS) by first adding DPBS to the side of the dishes to avoid disturbing the cell layer, and manually rock the dishes back and forth several times.
2. Remove and discard the DPBS using a suction and add 2 mL of 0.25% (w/v) trypsin diluted in DPBS, and gently rock the dishes to cover the cell layer. Put the dishes into a CO_2 incubator (37 °C, humidified air, 5% CO_2) for 5 min.
3. After pipetting up and down using a disposable pipette to break up detached cells, collect the cell suspension into a 15 mL tube and measure the cell density using a hematocytometer under a microscope[18].
4. Dilute the cells to 2.5 × 10^5 cells/mL with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Add 2 mL (5 × 10^5 cells) of the diluted cell suspension to each well of 6-well plates.
5. Grow the cells overnight in a CO_2 incubator (37 °C, humidified air, 5% CO_2).
6. **Transfection into HEK293 cells**
   NOTE: If phosphorylation of endogenous Rab10 is to be examined, proceed to step 1.7. Plasmids used in this protocol can be obtained from the authors upon request. See **Table of Materials** for brief information and **Figure S1** for their DNA sequences.
   1. Aliquot 200 µL of DMEM into 1.5 mL tubes.
   2. To each tube, add both 0.266 µg (final concentration of 1.33 µg/mL) of a plasmid encoding HA-Rab10 and 1.066 µg (final concentration of 5.33 µg/mL) of a plasmid encoding 3×FLAG-LRRK2 from 500 µg/mL plasmid stocks. Then add 4 µL of 1 mg/mL solution of polyethyleneimine (dissolved in 20 mM HEPES-NaOH, pH 7.0) and immediately mix the solution by vortexing for 5 s.
   3. Let the tubes stand at room temperature for 10 min and add the content of one tube dropwise to one well using a micropipette. Gently and manually rock the culture plates back and forth several times to let the transfection mixture diffuse evenly throughout the well.
7. Let the cells grow for another 24-36 h in a CO_2 incubator (37 °C, humidified air, 5% CO_2).
8. If phosphorylation of endogenous Rab10 is to be examined, treat cells with and without LRRK2 inhibitors for 1 h before lysing cells.
   1. Prepare stock solutions of LRRK2 inhibitors by dissolving the inhibitors in dimethyl sulfoxide (DMSO) at 10 mM. We recommend using MLi-2 and GSK2578215A, which are highly specific and potent LRRK2 inhibitors. Store stock solutions at -80 °C.
   2. Prepare working stocks of the inhibitors by diluting the stock solutions with DMSO: For MLi-2, prepare 10 µM and 30 µM for endogenous and overexpressed LRRK2, respectively. For GSK2578215A, prepare 1 mM and 3 mM for endogenous and overexpressed LRRK2, respectively.
   3. Add 2 µL of the working stocks of either MLi-2 or GSK2578215A to the middle of one well using a micropipette and gently rock the plate manually back and forth several times to let the inhibitors diffuse evenly throughout the well.
   4. Add 2 µL of DMSO to a different well as a negative control in a similar manner to the inhibitor in step 1.8.3.
   5. Put the plates back to the incubator and culture the cells for 1 h.
9. Lyse the cells.
   1. Put the culture plates on ice. Remove and discard the media. Wash the cells by first adding 2 mL DPBS to the side of the dishes to avoid disturbing the cell layer and manually rock the dishes back and forth several times.

2. Remove and discard the DPBS, and add to the cells 100 µL of the lysis buffer (50 mM Tris-HCl pH 7.5, 1% (v/v) polyoxyethylene(10) octylphenyl ether, 1 mM EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid), 1 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 0.1 µg/mL microcystin-LR, 270 mM sucrose, protease inhibitor cocktail).
3. Tilt the plates on ice and scrape the cells using a cell scraper to gather as much cell lysate as possible. Collect the lysates using a micropipette into 1.5 mL tubes (pre-chilled on ice).
   Caution: Microcystin-LR can be fatal if swallowed or in contact with skin.

10. Let the tubes stand on ice for 10 min for complete lysis. Clarify the cell lysates by centrifugation (20,000 × g, 10 min at 4 °C) and transfer the supernatants to new 1.5 mL tubes pre-chilled on ice.
11. Measure protein concentration (µg/µL) of the cleared lysates by Bradford assay.
    1. Prepare bovine serum albumin (BSA) standards (0.2, 0.4, 0.6, 0.8, and 1 mg/mL) by diluting the stock solution with distilled water. Dilute the cleared cell lysates by 20-fold with distilled water.
    2. Put 5 µL/well of the BSA standards, blank (distilled water), and each diluted cell lysate into a 96-well plate in triplicate.
    3. Add 150 µL/well of the Bradford assay reagent using 12-channel micropipette and let the plate stand at room temperature for 5 min.
    4. Measure the absorbance at 595 nm on a plate reader and compare to BSA standards.

12. Prepare 100 µL of samples for SDS-PAGE. The protein concentration of the samples is 1 µg/µL for overexpressed HA-Rab10 and 2 µg/µL for endogenous Rab10.
    1. Using the quantified concentrations from step 1.11.4, calculate the volume (µL) of the cell lysates equivalent to 100 µg (overexpressed HA-Rab10) or 200 µg (endogenous Rab10) by dividing the protein amount (100 µg or 200 µg) by the protein concentration of lysates (µg/µL).
    2. Add 25 µL of 4× Laemmli's SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 4% (v/v) β-mercaptoethanol) to new 1.5 mL tubes kept at room temperature.
    3. Add the calculated volume of the cell lysates to each tube and mix by vortexing for 5 s at room temperature.
    4. Bring the total volume to 100 µL with the lysis buffer and mix by vortexing for 5 s at room temperature.

13. Supplement with 10 mM $MnCl_2$ to quench the chelating agent. Add 1 µL of 500 mM $MnCl_2$. Mix by vortexing for 5 s at room temperature.
    NOTE: Samples containing $MnCl_2$ may not be suitable for normal SDS-PAGE. This step is required due to the presence of the chelating agent (such as ethylenediaminetetraacetic acid (EDTA), EGTA, *etc.*) in the lysis buffer. Otherwise, Mn^{2+} ions coupled to P-tag acrylamide will be dissociated by the chelating agents, and P-tag SDS-PAGE will not work properly.
14. Boil all samples at 100 °C for 5 min and store the samples below -20 °C until use. The boiled samples can be stored up to at least 6 months.

2. Casting Gels for P-tag SDS–PAGE

NOTE: Gels should be made on the same day as running the gels. Gels can be made under ambient light conditions.

1. Prepare 5 mM P-tag acrylamide stock solution by first dissolving the 10 mg of powder/solid P-tag acrylamide completely with 100 µL of methanol and then bring to 3.3 mL by adding double distilled water.
   NOTE: P-tag acrylamide is light sensitive. The prepared solution should be stored in the dark at 4 °C until use.
2. Clean both plain and notched plates by spraying 70% ethanol and wiping with a paper towel. Assemble the gel plates. The dimensions of the gel plates used in this particular protocol are 80 or 100 mm long by 100 mm wide. Clean silicon spacers are put in between plain and notched plates and the assembled plates are clamped with binder clips.
   NOTE: Any type of gel plates that work for ordinary SDS-PAGE can be used.
3. Put a comb to be used (17-well plastic comb) into the assembled gel plates and mark on the gel plate the position of the bottom of the wells with a permanent marker.
4. Prepare 10 mL of 10% acrylamide gel mixture (10% (w/v) acrylamide (acrylamide:bis-acrylamide = 29:1), 375 mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS) in a 15 mL tube.
   NOTE: The optimal concentration of acrylamide may vary depending on the reagents used. Tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) should not be added at this point.
5. Add 100 µL of 5 mM P-tag acrylamide and 10 µL of 1 M $MnCl_2$ solutions at final concentrations of 50 µM and 100 µM, respectively.
   NOTE: The optimal concentrations of the P-tag acrylamide and $MnCl_2$ might also vary depending on the reagents used.
6. Add 15 µL of TEMED to the gel mixture at a final concentration of 0.15% (v/v) and then 50 µL of 10% (w/v) APS at a final concentration of 0.05% (w/v). Mix well by gently swirling the tube for 5 s and pour into the assembled plates immediately up to a height that is 2 mm below the position marked in step 2.3.
7. Gently layer 2-propanol onto the gel solution to flatten the top of separating gels.
8. Let the gels stand for 30 min at room temperature. It is not necessary to protect the gels from light.
   NOTE: It might take longer for gels to set under cold room temperature. Degassing the gel mixture before adding TEMED and APS helps accelerate this step. For this purpose, the gel mixture can be prepared in a 100-200 mL Erlenmeyer flask connected to a suction. Degas the solution for 10 min.
9. Remove the layered 2-propanol by absorbing with a paper towel.
10. Wash the top space of the gels by filling the space up with distilled water from a wash bottle and discard the water by pouring off into basin. Repeat washing 3 times.
11. Remove residual water remaining in the top space of the gels by absorbing with a paper towel.
12. Prepare 3 mL of 4% acrylamide gel mixture (4% (w/v) acrylamide (a: crylamide:bis-acrylamide = 29:1), 125 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS) in a 15 mL tube.
    NOTE: Do not add P-tag acrylamide or $MnCl_2$ solution to the stacking gel mixture.
13. Add 7.5 µL of TEMED and 24 µL of 10% (w/v) APS at final concentrations of 0.25% (v/v) and 0.08% (w/v), respectively. Mix well by gently swirling the tube for 5 s and pour the mixture on top of the separation gel. Immediately put appropriate combs (17-well plastic combs which accommodate up to 25 µL of samples, for example).

14. Let the gels stand for 30 min at room temperature. It is not necessary to protect the gels from light. After the stacking gels set, run them without further storage.

3. SDS–PAGE and Immunoblotting

1. Remove the combs from the gels. Then remove the silicon spacers and then clips from the gels.
2. Put the casted gels into gel tanks and fix the gel to the tank by clamping with the binder clips.
3. Pour the running buffer (25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS) at the bottom and top of the gels. Clean wells by flushing the running buffer using a 5 mL syringe and a 21G needle to remove gel pieces.
4. Remove air bubbles from the bottom space of the gels using a bent needle attached to a syringe. To make a bent needle, manually bend a 21G needle in the middle of the needle so that the angle between the tip and the base of the needle becomes 30-45 °.
5. Spin down precipitates caused by the addition of $MnCl_2$ at 20,000 × g for 1 min at room temperature to obtain clear samples.
6. Load 10 µg proteins for detecting the phosphorylation of overexpressed Rab10 and 30 µg proteins for endogenous Rab10.
   NOTE: It is critical to load equal volume of samples in ALL wells. Empty lanes should be loaded with 1× Laemmli's SDS-PAGE sample buffer. If the samples contain $MnCl_2$, add the same concentration of $MnCl_2$ to the dummy samples.
7. The well loaded with a molecular weight marker (MWM) should also be supplemented with 1× Laemmli's SDS-PAGE sample buffer so the volume of samples loaded is the same in all wells.
   NOTE: Again, if the samples contain $MnCl_2$, add the same concentration of $MnCl_2$ to the MWM. Alternatively, an EDTA-free MWM can be used.
8. Run gels at 50 V for stacking (approximately 30 min) until the dye front crosses into the separation gel.
9. After the samples stack, change the voltage to 120 V for separation until the dye front reaches the bottom of the gels (approximately 50 and 80 min for 80 and 100 mm long gels, respectively).
   NOTE: The migration pattern of the MWM is expected to be different from that on normal SDS-PAGE gels. It should not be used for estimating the molecular weight of proteins on P-tag SDS-PAGE gels but can be used for checking the reproducibility of P-tag gels. Refer to **Discussion** for details.
10. Wash the gels to remove $MnCl_2$ from the gels.
    1. Pour the transfer buffer (48 mM Tris, 39 mM Glycine, 20% (v/v) methanol) containing 10 mM EDTA and 0.05% (w/v) SDS into a container (*e.g.*, large weighing boats). The volume of the buffer should be sufficient to cover a gel.
    2. Remove the separation gels from the gel plates and put one gel in one container. Discard the stacking gel.
    3. Leave the gels on a rocking shaker (~60 rpm) for 10 min at room temperature.
    4. Repeat the wash steps in total 3 times.
       NOTE: Use fresh buffer for each wash.
    5. Wash the gels once for 10 min with the transfer buffer containing 0.05% (w/v) SDS to remove EDTA. The volume of the buffer should be sufficient to cover a gel.

11. Electro-transfer to nitrocellulose or polyvinylidene difluoride (PVDF) membranes using wet tanks.
    1. Place a filter paper (10 x 7 cm) on a sponge pad for transfer. Place the gel on the filter paper. Make sure there are no air bubbles between the filter paper and the gel.
    2. Put a membrane (10 x 7 cm) on the gel and make sure there are no air bubbles between the gel and the membrane.
    3. Put another filter paper (10 x 7 cm) on the membrane and, again, make sure there are no air bubbles between the membrane and the filter paper.
    4. Put another sponge pad on the filter paper. Put the stack of membrane/filter papers in a cassette for transfer.
    5. Put the cassette into a transfer tank, making sure that the membrane is located between the gel and the positively charged anode.
    6. Connect the tank to a power supply and put the tank in a styrene foam box filled with ice-cold water. Start transfer at 100 V for 180 min.
       NOTE: Prolonged transfer is necessary since the transfer of proteins from P-tag SDS-PAGE gels are not as efficient as that from normal SDS-PAGE gels. Efficient cooling is essential to avoid melting gels during transfer.

12. **Check the transfer**
    1. Remove the membranes from the gels using tweezers and soak the membranes in a Ponceau S solution (0.1% (w/v) Ponceau S, 5% (v/v) acetic acid) to stain transferred proteins on the membranes in a plastic container. The volume of the solution should be sufficient to cover a membrane.
    2. Incubate the membranes for 1 min at room temperature by rocking manually.
       NOTE: A ladder of bands should become visible in each lane.
    3. Pick the membrane up out of the staining solution with tweezers and see if the ladder of bands has uniform pattern and intensity in every lane where the samples have been loaded.
    4. After checking the staining, remove the staining solution and add TBST buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% (v/v) polyoxyethylenesorbitan monolaurate). The volume of the buffer should be sufficient to cover a membrane.
       NOTE: The Ponceau S solution can be collected and re-used several times.
    5. Rock the membranes in TBST on a rocking shaker (~60 rpm) at room temperature until no visible bands remain on the membranes.
    6. Repeat the washing step with fresh TBST for 5 min.

13. Remove and discard the TBST. Block by adding 5% (w/v) skim milk dissolved in TBST and rocking on a shaker (~60 rpm) for 1 h at room temperature. The volume of the blocking solution should be sufficient to cover a membrane.
14. Prepare primary antibody solutions by diluting primary antibodies (anti-Rab10 antibody for endogenous Rab10 and anti-HA antibody for overexpressed HA-Rab10) in 10 mL per membrane of the blocking solution (see **Table of Materials** for concentrations).
15. Remove and discard the blocking solution and add the primary antibody. Incubate the membranes on a rocking shaker (~60 rpm) overnight at 4 °C.
16. Remove the primary antibody solution and add TBST to wash the membranes. The volume of the buffer should be sufficient to cover a membrane.

17. Incubate the membranes for 5 min on a rocking shaker (~60 rpm) at room temperature. Repeat the wash (step 3.16) in total 3 times using fresh TBST each time.
18. Prepare secondary antibody solutions by diluting secondary antibody labeled with horseradish peroxidase (HRP) in 10 mL per membrane of the blocking solution. Use anti-rabbit IgG antibody labeled with HRP for membranes probed with the anti-Rab10 antibody, and anti-mouse IgG antibody labeled with HRP for those probed with the anti-HA antibody (see **Table of Materials** for concentration).
19. Remove and discard the TBST after the third wash and add the secondary antibody solution. Incubate the membranes on a rocking shaker (~60 rpm) for 1 h at room temperature.
20. Wash the membranes in TBST for 10 min. Repeat the wash step in total 3 times, similar to step 3.17.
21. Develop the membranes using enhanced chemiluminescence (ECL).
    NOTE: Exposure time might vary depending on the ECL solution and the system used for detection of chemiluminescence.
    1. Turn on an imager equipped with a charge-coupled device (CCD) camera and a computer connected to the imager. Start up the control software for the imager. Wait until the temperature of the CCD camera has reached -25 °C.
    2. Put 1 mL of the ECL solution for one membrane on a plastic wrap spread on a bench.
    3. Put a membrane gel-side-up on the ECL solution and then quickly flip it over so that both sides of the membrane are coated with the solution.
    4. Pick the membrane up and drain it by letting one side of the membrane touch a paper towel for 5 s.
    5. Put the membrane between clear films (*e.g.*, clear paper pockets).
       NOTE: Plastic wrap is not recommended for wrapping the membranes. The clear films used for wrapping the membrane need to be as flat as possible without visible wrinkles to avoid uneven background.
    6. Place the membrane on a black tray. Put the tray in the imager and close the door.
    7. Click the "Focusing" button in the window of the control software. Check that the membrane is correctly positioned. Click the "Return" button.
    8. Select "Precision" for "Exposure Type". Select "Manual" for "Exposure Time" and set the exposure time to 1 s.
    9. Select "High" for "Sensitivity/Resolution". Leave "Add Digitization Image" unchecked. Click the "Start" button to take an image.
    10. Save the image that appeared on the display in the computer as a TIFF file.
    11. Repeat taking images with the exposure time from 1, 3, 10, 30, 60, 90, 120, 150 s and up to 180 s. When taking the last image, check "Add Digitization Image" so the digital image, not the chemiluminescence, of the membrane can be simultaneously taken.
    12. Select the best image with the largest dynamic range and without any saturating pixels (shown in red) in the bands of interest.
        NOTE: Conventional X-ray films can also be used for detection[19].

Representative Results

Overexpression System: Phosphorylation of HA-Rab10 by 3×FLAG-LRRK2 in HEK293 Cells:

HEK293 cells were transfected with 0.266 µg of HA-Rab10 wild-type and 1.066 µg of 3×FLAG-LRRK2 (wild-type, kinase-inactive mutant (K1906M), or FPD mutants). Rab10 phosphorylation was examined by P-tag SDS-PAGE followed by immunoblotting using an anti-HA antibody (**Figure 2**). 10 µg of proteins were run on a 10% gel (80 x 100 x 1 mm) containing 50 µM P-tag acrylamide and 100 µM $MnCl_2$. The exposure time for the P-tag gel (top panel) was 10 s using a standard ECL solution. Co-overexpression of LRRK2 with Rab10 caused band shift on the P-tag gel (marked with an open circle in the top panel; compare lanes 2 and 4). In contrast, co-expression with a kinase-inactive mutant (K1906M) LRRK2 failed to change the mobility of Rab10 (compare lanes 4 and 5), indicating that the band shift is due to the LRRK2 kinase activity. The band shift was increased by all FPD mutations (compare lanes 4 and 6-13) in agreement with the previous report[15].

Endogenous System: Phosphorylation of Rab10 by LRRK2 in Cultured Cells:

Mouse 3T3-Swiss albino embryonic fibroblast cells were treated with or without a LRRK2 inhibitor (GSK2578215A)[20] at a final concentration of 1 µM for 1 h (**Figure 3A**). Human lung carcinoma A549 cells were treated with or without another LRRK2 inhibitor (MLi-2)[21] at a final concentration of 10 nM for 1 h (**Figure 3B**). Endogenous Rab10 phosphorylation by endogenous LRRK2 was examined by P-tag SDS-PAGE followed by immunoblotting with an anti-Rab10 antibody. 30 µg of proteins were run on a 10% gel (100 x 100 x 1 mm for 3T3-Swiss albino cells and 80 mm for A549 cells) containing 50 µM P-tag acrylamide and 100 µM $MnCl_2$. The exposure for the P-tag gel (top panel) in **Figure 3A** and **Figure 3B** were 3 min and 90 s, respectively. The band shift corresponding to phosphorylated endogenous Rab10 was observed on the P-tag gel (marked with an open circle in the top panel; compare lanes 1-2 and 3-4) which disappeared upon treatment of cells with GSK2578215A or MLi-2. The efficacy of the LRRK2 inhibitors was validated by immunoblotting using the anti-pSer935 LRRK2 antibody (the third panel from the bottom), which is a well-established readout of LRRK2 inhibition in cells[22].

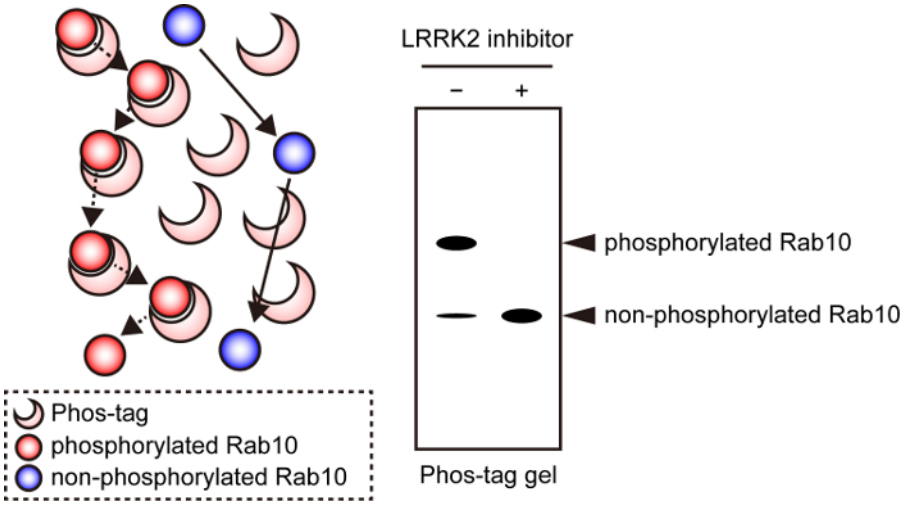

Figure 1. Schematic depiction of P-tag SDS–PAGE. When a mixture of phosphorylated (red circles) and non-phosphorylated (blue circles) proteins (*e.g.*, Rab10) runs through a gel where P-tag acrylamide is co-polymerized, the mobility of only phosphorylated proteins retards due to the strong interaction of phosphorylated proteins with the P-tag molecules (broken arrows). Since Rab10 is phosphorylated by LRRK2 at a single residue Thr73, Rab10 runs as two bands: the top band represents phosphorylated Rab10 and the bottom band represents non-phosphorylated Rab10. When cells are treated with LRRK2 inhibitors, the top band should disappear. Please click here to view a larger version of this figure.

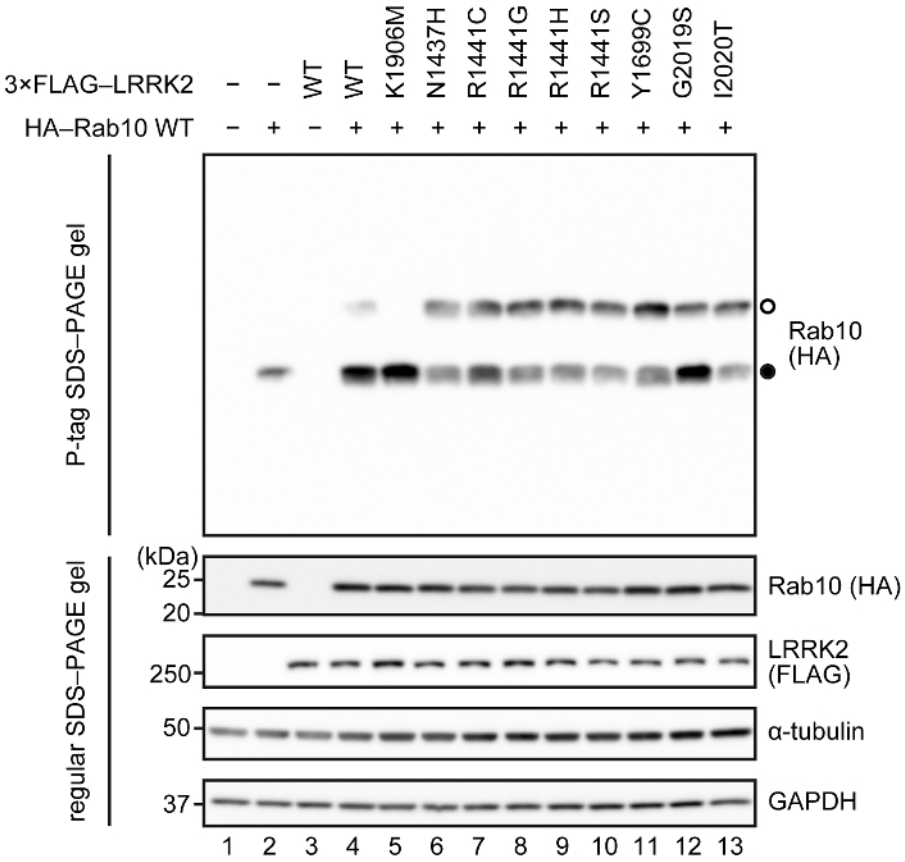

Figure 2. Representative result of the P-tag blot: overexpression system. The top panel shows the P-tag blot using an anti-HA antibody, where phosphorylated and non-phosphorylated Rab10 are marked with open (○) and closed (●) circles, respectively. The panels shown below the P-tag blot are immunoblots on normal SDS-PAGE gels using an anti-HA, anti-FLAG, anti-α-tubulin, and anti-GAPDH antibodies. The HA blot and FLAG blot are shown for ensuring the overexpression of HA-Rab10 and 3xFLAG-LRRK2, respectively. The α-tubulin and GAPDH blots are shown for ensuring the equal loading. Please click here to view a larger version of this figure.

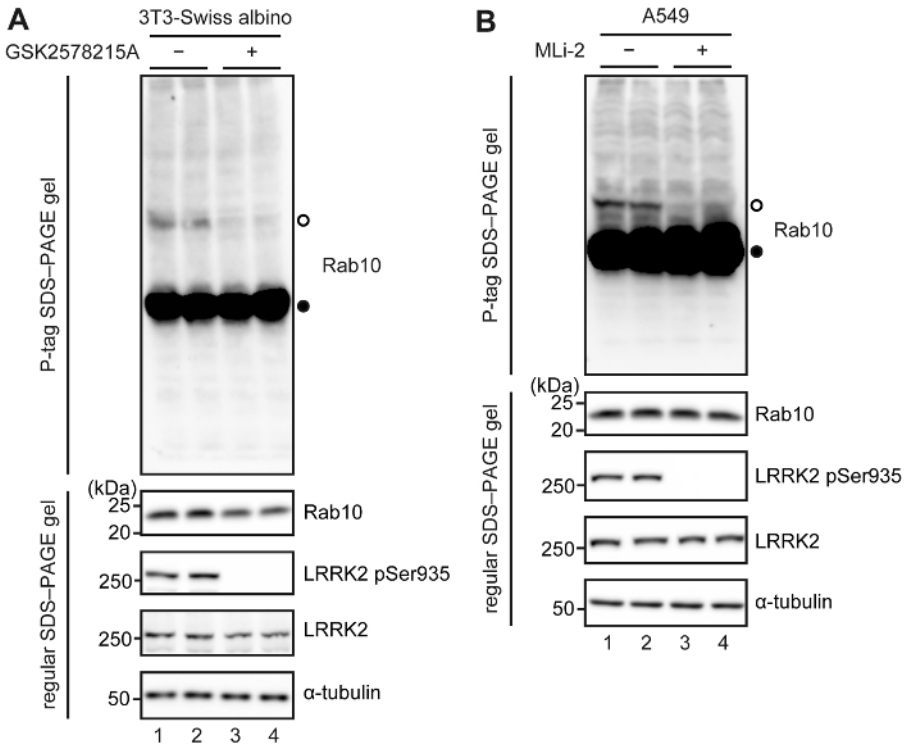

Figure 3. Representative result of the P-tag blot: endogenous system. Two cell lines, namely (**A**) 3T3-Swiss albino cells and (**B**) A549 cells, were used. In both figures, the top panels show the P-tag blots using an anti-Rab10 antibody, where phosphorylated and non-phosphorylated endogenous Rab10 are marked with open (○) and closed (●) circles, respectively. The panels shown below the P-tag blots are immunoblots on normal SDS-PAGE gels using an anti-Rab10, anti-pSer935 LRRK2, anti-LRRK2, and anti-α-tubulin antibodies. The Rab10 and LRRK2 are shown for ensuring the similar expression of endogenous Rab10 and LRRK2 between the samples, respectively. The pSer935 LRRK2 blot are shown for ensuring that GSK2578215A (**A**) and MLi-2 (**B**) worked as expected. The α-tubulin blot are shown for ensuring the equal loading. The images of the P-tag blots superimposed with the molecular weight marker are shown in **Figure S4**. Please click here to view a larger version of this figure.

Figure S1. DNA sequences of the plasmids. The DNA sequences of the plasmid encoding HA-Rab10/pcDNA5 FRT TO and that of 3×FLAG-LRRK2/p3×FLAG-CMV-10. All plasmids used in this protocol are available from the authors upon request. Please click here to download this file.

Figure S2. An example of the optimization of P-tag SDS-PAGE. The same set of samples as that used in **Figure 3A** was used for optimizing the P-tag SDS-PAGE. Four different conditions were tested as shown in the figure. The bands corresponding to phosphorylated and non-phosphorylated Rab10 are highlighted with solid and dashed line rectangles, respectively. Based on this experiment, the condition with 10% acrylamide, 50 μM P-tag acrylamide and 100 μM $MnCl_2$ gave the best separation of the two bands, both locating in the middle of the gel. Please click here to download this file.

Figure S3. Comparison of the migration pattern of a molecular weight marker. The molecular weight marker (MWM) was run on a regular SDS-PAGE gel (left panel) or on a P-tag SDS-PAGE gel (middle panel), and the gels were stained by Coomassie staining. The right panel shows an immunoblot of the samples used in **Figure 2** (lane 4 and 5) on the same P-tag SDS-PAGE gel as the middle panel to show the positions of phosphorylated and non-phosphorylated Rab10. The arrowhead on the right-hand side of the immunoblot indicates the position of one of the MWM on the P-tag SDS-PAGE gel. Please click here to download this file.

Figure S4. The position of a molecular weight marker on P-tag SDS-PAGE gels. Digitized images of the membranes used for **Figure 3A** and **Figure 3B** are shown as (**A**) and(**B**), respectively. The immunoblots shown in **Figure 3** are superimposed to show the relative position of the molecular weight marker (MWM). The MWM did not transfer well to the membranes but the marker (arrowhead) in **Figure S3** was faintly but consistently observed. The position of the MWM is marked with dotted lines. Note that lanes irrelevant to the figures between the MWM and the lanes of interest are crossed out. Please click here to download this file.

Discussion

Here, we describe a facile and robust method of detecting Rab10 phosphorylation by LRRK2 at endogenous levels based on the P-tag methodology. Because the currently available antibody against phosphorylated Rab10 works only with overexpressed proteins[15], the present method utilizing P-tag SDS-PAGE is the only way to assess endogenous levels of Rab10 phosphorylation. Moreover, the present method allows the estimation of the stoichiometry of Rab10 phosphorylation in cells. Because the P-tag methodology is generically applicable to phospho-proteins, the present protocol can be a "prototype" for establishing similar methods for other phospho-proteins.

Critical steps in the protocol are casting gels and preparation of samples. P-tag acrylamide is a relatively photo-labile reagent and the ability to retard the electrophoretic mobility of phosphorylated proteins is sometimes lost after long term storage at 4 °C. Researchers should take every

care to avoid exposing P-tag acrylamide to light. Moreover, the P-tag molecule needs to form a complex with Mn^{2+} ions to capture phosphates. Thus, samples should not contain chelating agents such as EDTA, which are usual components of commercially available SDS-PAGE sample buffers. We recommend using the classical Laemmli's sample buffer to prepare samples for P-tag SDS-PAGE.

Another critical point is to thoroughly optimize the concentrations of acrylamide, P-tag acrylamide and $MnCl_2$ in the separation gel mixture (**Figure S2**). The migration distances of phosphorylated and non-phosphorylated bands will vary depending on the reagents used (lot, purity, *etc.*), and optimization of the proper concentrations by testing several different concentrations in combination is mandatory (*e.g.,* P-tag acrylamide (25, 50, 75 μM), $MnCl_2$ (1:2 or 1:3 molar ratio to P-tag acrylamide), and acrylamide (7.5, 10, 12.5%)). The phosphorylated and non-phosphorylated bands should appear in the middle of the gels and well separated from each other. For optimization process, it is recommended to use overexpressed Rab10 because the shifted band is readily detectable. The authors can provide control samples for this protocol.

One of the biggest confusion that this protocol can cause will be due to the difference of the migration patterns of the MWM between regular and P-tag SDS-PAGE gels. As shown in **Figure S3**, the migration patterns of the MWM on regular and P-tag SDS-PAGE gels (both 10%) are greatly different and one cannot use the MWM for estimating the molecular weight of proteins on P-tag SDS-PAGE gels. However, on P-tag SDS-PAGE gels, one of the MWM stands out by migrating to the middle of the gel (arrowhead in **Figure S3**), which is consistently observed among gels (see **Figure S4**). This MWM always migrates in between the bands of phosphorylated and non-phosphorylated Rab10. Therefore, this marker is useful not only for ensuring that P-tag SDS-PAGE works before transfer but also as a landmark for having a rough sense of where the bands of phosphorylated and non-phosphorylated Rab10 will be seen.

For detection of bands on immunoblots, we have used an imager equipped with a cooled CCD camera (see **Table of Materials**) as well as a conventional X-ray film developing system, and found that both systems work well. For detection of endogenous levels of Rab10 phosphorylation in cultured cells, it is necessary to use cell lines which have high expression levels of endogenous LRRK2, such as mouse embryonic fibroblasts (either primary cultured or immortalized cell lines (3T3-Swiss albino, *etc.*)) and human lung carcinoma-derived A549 cells. For detection of endogenous levels of Rab10 phosphorylation in mouse tissues, it is recommended to use lung[16].

The quantitation of Rab10 phosphorylation is not beyond that of usual immunoblotting. If the stoichiometry of Rab10 phosphorylation is very low (as shown in **Figure 3**), the intensity of non-phosphorylated band (marked with a closed circle) tends to be far above the saturation level, making the precise determination of the stoichiometry impossible. Nevertheless, qualitative estimation is still possible in any case, which is not obtainable without using the present method. Since the detection of the phosphorylation of endogenous Rab10 requires prolonged exposure, there tends to be some nonspecific bands appearing on the P-tag blot even though the anti-Rab10 antibody used in this protocol gives fairly clean immunoblots for normal use. To distinguish phosphorylated proteins from nonspecific bands, it is critical to use an inhibitor-treatment control, in which phosphorylated bands, but not nonspecific bands, should disappear. LRRK2 phosphorylates Rab8 besides Rab10[15], and we have successfully applied the present protocol to Rab8A as well (data not shown). The present protocol can potentially be used to examine the phosphorylation of any proteins, although there might be technical difficulties if the protein is large (>100 kDa) or multiply phosphorylated. Because Rab10 is a small protein (25 kDa) and singly phosphorylated at Thr73 by LRRK2, the result of P-tag SDS-PAGE is simple where only one shifted band is observed.

In the near future, it will be critical to establish the method to quantitatively detect the kinase activity of LRRK2 in patient-derived samples in a high-throughput manner to assess the alteration of the kinase activity of LRRK2 in PD patients as well as to evaluate the effect of drugs on LRRK2 in clinical trials. Since human peripheral blood mononuclear cells (PBMCs) express relatively high levels of endogenous LRRK2[23], PBMCs or further isolated blood cells will be worth testing for endogenous Rab10 phosphorylation. The present method will not only be useful in investigating the basic biology of the LRRK2 signaling pathway, but also aid in obtaining a basic proof-of-concept for deciding which sample in patient-derived samples is to be analyzed in such large-scale studies.

Disclosures

The authors have nothing to disclose.

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