1. Preparing the kinase reactions

1. Thaw protein on ice - LRRK2 wild type, D1994A, G2019S.

Safety precautions relevant to this protocol have been noted in the text, highlighted with the radioactive trefoil symbol (放射性ヨシ). Users in other countries should confirm local rules, regulations and licensing authorities with their radiation safety officer. Canada the Canadian Nuclear Safety Commission (http://nuclearsafety.gc.ca/eng/), and in Germany Das Bundesamt für Strahlenschutz (http://ionising/index.htm), in the United States the Nuclear Regulatory Commission (http://www.nrc.gov/materials/miau/regs-guides-comm.html), in the United Kingdom, the Health and Safety Executive (http://www.hse.gov.uk/radiation/ionising/index.htm), in the United States the Nuclear Regulatory Commission (http://www.nrc.gov/materials/miau/regs-guides-comm.html), in Canada the Canadian Nuclear Safety Commission (http://nuclearsafety.gc.ca/eng/), and in Germany Das Bundesamt für Strahlenschutz (http://www.bfs.de/de/bfs). Users in other countries should confirm local rules, regulations and licensing authorities with their radiation safety officer. Safety precautions relevant to this protocol have been noted in the text, highlighted with the radioactive trefoil symbol (放射性ヨシ).
2. **Running the assay**

   - All steps utilizing $^{32}$P ATP should take place in designated radiation areas.
   - Suitable personal protective equipment should be worn - under standard operating procedure in our laboratory these include lab coat, double gloves and protective goggles.
   - Samples containing $^{32}$P ATP should be shielded from users by 6mm Perspex screens to minimize exposure.
   - Where applicable, personal monitoring devices should be used - within UCL, any certified open source radiation user must have a film badge to monitor radiation exposure during experiments.
   - All experimental surfaces should be assessed for radioactive contamination before and after use using a Geiger counter.

   1. Prior to starting assay, set heating blocks to 30°C and 100°C respectively.
   2. Remove $^{32}$P ATP from -20 freezer (note that storage conditions for $^{32}$P ATP may vary depending on supplier or type of radionucleotides used). Scan outside of container prior to use, thaw behind perspex screen.
   3. With reactions on ice, add 1μl of $^{32}$P ATP to each along with 10μM of cold ATP.
   4. Mix well with pipette. Pulse centrifuge to bring liquid to bottom of tube, minimizing risk of contamination.
   5. Remove 15μl aliquot for zero time point and terminate reaction in aliquot by addition of 5μl of 4x SDS sample buffer and denaturation at 100°C for 10 minutes. Pulse centrifuge to bring liquid to bottom of tube, minimizing risk of contamination.
   6. Remaining sample placed in heating block and incubated at 30°C for 60 minutes.
   7. 15μl removed at 60 minute time point and reaction terminated by addition of 5μl of 4x SDS sample buffer and denaturation at 100°C for 10 minutes. Pulse centrifuge to bring liquid to bottom of tube, minimizing risk of contamination.

3. **Immunoblotting the samples and analysing results**

   1. **Samples run on SDS-PAGE**
      1. 10 well 4-12% Bis-tris polyacrilamide gel prepared for electrophoresis using MOPS running buffer.
      2. 20μl of each sample loaded onto gel along with 7μl of sharpstain protein standard ladder.
      3. Gel run at 160v for 90 minutes, or until dye front has reached the end of the gel. All liquid in contact with radioisotopes should be treated as radioactive waste and disposed of as per institutional guidelines. UCL regulations state that liquid radioactive waste should be discarded by pouring down designated radioactive disposal sinks with copious water.

   2. **Protein transferred to PVDF membrane via western blot**
      1. Transfer buffer prepared, 1x Tris Glycine plus 20% Methanol. PVDF Membrane and filter paper cut to correct size for gel and activated with glacial methanol in the case of the membrane or pre-wet with transfer buffer in the case of the filter paper. The membrane should be pre-labelled with ballpoint ink to allow identification of orientation. Gel removed from plastic casing and excess acrylamide removed and disposed of as radioactive waste.
      2. The gel should be formed into a sandwich with the membrane and filter paper, ensuring that no bubbles exist between the membrane and gel, and then arranged in the western blot apparatus with the membrane between the gel and the anode.
      3. Transfer carried out at 25V for 16 hours.

   3. Following protein transfer to PVDF, the membrane should be dried at room temperature. Finally, the dry membrane should be isolated between cellulose acetate sheets and exposed to either a phosphor screen or x-ray film to allow detection of radiolabelled protein. Exposure time can run from several hours to over a week depending on the specific activity of the radioisotope used and the enzyme kinetics of the reaction.

4. **Representative Results**

   Figure 1 shows representative results for an assay carried out using wild type, G2019S and D1994A LRRK2 with Myelin Basic Protein as a generic phosphate acceptor substrate. Autophosphorylation of LRRK2 is visible at 200kDa, with multiple bands representing phosphorylated MBP visible from 20-40kDa. Note the absence of autophosphorylation in the D1994A (kinase dead) lane, and increased phosphorylation due to the G2019S mutation. Note also residual phosphorylation of MBP in the kinase dead lane. This may be due to incomplete ablation of the kinase activity of LRRK2 by the D1994A mutant or reflect the presence of trace contaminating kinases in the reaction.
Discussion

This paper describes a basic protocol for assaying the kinase activity of LRRK2 using an in vitro system. In the interests of brevity, this has been limited to a one-hour end point template using a generic substrate, but the general protocol is applicable to a range of potential substrates and amenable to more sophisticated analyses examining the kinetics of the kinase activity of LRRK2. This highlights one of the key advantages of using an in vitro system to examine the kinase behaviour of a protein such as this: because there is complete control over the concentrations of the enzyme and substrate, it is possible to generate kinetic data and calculate \( K_m \) and \( V_{max} \) values for the reaction. For more detailed kinetic evaluations or evaluating the impact of putative inhibitors, a higher throughput system (such as that afforded by using the LRRKtide substrate, available from Invitrogen) is a superior alternative to the approach described here.

It is important, however, to recognise that the reductionist model system provided by in vitro kinase assays is but one tool to examine the biology of a kinase and its relationships with potential substrates. Data from assays such as this should be used in conjunction with other approaches to gain a comprehensive picture of how a kinase behaves in vitro and in a cellular context. For example, a putative substrate phosphorylated in vitro can be analysed by mass spectrometry to identify possible phosphosites that can then be manipulated by targeted mutagenesis (e.g. converting serine or threonine phosphate acceptor residues to none-phosphorylatable residues such as alanine) to examine the functional consequences of phosphorylation ex vivo.

A key consideration in interpreting data from an in vitro assay system such as this is likelihood of either type I or type II (false positive or false negative) errors. The reductionist nature of this system unfortunately disposes it to both - in the case of the former, having a purified putative substrate and purified kinase in artificially close proximity and at high concentration (compared to the cellular milieu) can result in artefactual phosphorylation events. Conversely, many kinases function as part of a complex within the context of the cell, and require co-factors for phosphorylation of a given substrate to occur. As noted above, a positive result from phosphorylation of a putative substrate using an in vitro assay system should then be tested in a cellular system to validate the result, and a negative result should be interpreted with caution.

In light of this, it is critical where possible to have both positive and negative controls to allow a comparative study of the activity of your kinase of interest. Careful selection of a positive control that has a known activity towards your protein of interest, along with a negative control that is unlikely to phosphorylate your putative substrate, is extremely valuable. One candidate control for LRRK2 is Receptor interacting kinase 3, which has a closely related primary sequence to the kinase domain of LRRK2 but has a very different overall domain structure. This is available as recombinant protein from Invitrogen and is used as a standard control in our laboratory.

It should also be noted that the commercially available form of LRRK2 lacks the N-terminus of the protein and is tagged with Glutathione-s-transferase and this should be taken into consideration as a potential confounding factor when carrying out kinase assays with this protein, as it is not known what role the N-terminal of LRRK2 may play in the normal function of this protein. If, for example, the N-terminal portion of LRRK2 that is absent in the protein used in this protocol is critical for recruitment of a specific substrate to the kinase domain of LRRK2 then this will have a major impact on the observed phosphorylation of said substrate in the in vitro system described above.

Even with these caveats, however, the importance attached to biology of LRRK2 in Parkinson's research highlights the utility of this protocol as a valuable tool for investigating the behaviour of this protein in an in vitro setting.

Disclosures

No conflicts of interest declared.

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