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Methods in Molecular Biology: MAP Kinase Signaling Protocols Book Proteomic Analysis of Scaffold Proteins in the ERK Cascade

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Abstract

ERK cascade scaffolds serve as docking platforms to coordinate the assembly of multi-protein complexes that contribute to the spatial and temporal control of ERK signaling. Given that protein-protein interactions are essential for scaffold function, determining the full repertoire of scaffold binding partners will likely provide new insight into the regulation and activities of the ERK cascade scaffolds. In this chapter, we describe methods to identify scaffold interacting proteins using a proteomics approach. This protocol is based on the affinity purification of scaffold complexes from tissue culture cells and utilizes mass spectrometry to identify the protein constituents of the complex.

Keywords

ERK cascade scaffolds; scaffold binding partners; proteomics; mass spectrometry; affinity purification; Pyo/Glu-Glu tag

1. Introduction

The ERK cascade is activated in response to many extracellular and intracellular cues and in collaboration with other signaling pathways, functions to transduce these signals into specific biological responses (1, 2). Like other MAPK cascades, the ERK cascade is a three-tiered kinase module, comprised of the Raf, MEK, and ERK protein kinases (3). In addition to these core components, a number of protein scaffolds have been identified that provide crucial spatial and temporal control of ERK cascade signaling (4, 5). In mammals, these proteins include MP-1, IQGAP, Paxillin, Sef, Kinase Suppressor of Ras 1 (KSR1), and KSR2 (6–11). By interacting with some or all of the core kinase components of the cascade, ERK scaffolds can increase the efficiency and specificity of ERK activation. These scaffolds can also recruit positive and negative regulators and coordinate feedback loops to modulate the intensity and duration of ERK cascade signaling. Moreover, an emerging theme for the ERK scaffolds is that through their distinct subcellular localizations, they can target the ERK module to specific cellular compartments and/or targets (12). Notably, each of these scaffold functions is mediated by the association of the scaffold with a particular set of proteins. Thus, determining the full repertoire of proteins that a scaffold interacts with will likely provide further insight into the regulation and activities of the ERK scaffolds.

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In this chapter, we describe a protocol for the analysis of ERK scaffold complexes using a proteomics approach. Our laboratory has successfully used this methodology to identify new binding partners for the KSR1 and KSR2 scaffolds, and further analysis of these interactions has revealed important regulatory mechanisms and functional properties of the KSR scaffold family (11, 13–15). This protocol involves the affinity purification of scaffold complexes from tissue culture cells and utilizes mass spectrometry to identify the protein constituents of the complex. Our laboratory exclusively uses the Pyoepitope tag (16, 17) for the affinity purification of protein complexes for mass spectrometry analysis. We find that the mouse monoclonal antibody recognizing the Pyo tag is highly specific and results in lower non-specific protein binding in comparison to other antibody/tag combinations such as FLAG, HA, or Myc-9E10. The methodology presented allows for the large scale and unbiased identification of scaffold binding partners and should prove useful for the study of other ERK cascade scaffolds, as well as the JIP family of scaffolds that regulate the p38 and JNK MAPK cascades.

2 Materials

Unless specifically noted, all solutions are prepared using deionized, distilled water and chemical reagents from Sigma-Aldrich (St. Louis, MO).

2.1. Generation of Pyo-Affinity Resin

1. Culture supernatant from hybridoma cells producing the Pyo monoclonal antibody.
2. Protein G Sepharose 4 Fast Flow affinity resin (GE Healthcare, Piscataway, NJ). Stored at 4°C.
3. Phosphate-buffered saline (PBS): 10 mM sodium phosphate buffer (pH 7.4), 137 mM NaCl, 1.5 mM KCl. Stored at 4°C.
4. 0.2 M sodium borate (pH 9.0), made fresh the day of use. To prepare, dissolve 3.81 g of sodium borate in 40 mL of water by warming to 60°C. Allow solution to cool to room temperature. If sodium borate precipitates out of solution, add a small volume of water, rewarm and cool completely. Adjust pH to 9.0 using concentrated HCl, and bring the final volume up to 50 mL with water.
5. Dimethyl pimelimidate (DMP).
6. 0.2 M ethanolamine (pH 8.0). To prepare, dissolve 1.95 g of ethanolamine in 75 mL of water, adjust the pH to 8.0 with 1N NaOH, and bring the final volume up to 100 mL with water.
7. 0.01% merthiolate in PBS. Note, merthiolate is very toxic and care should be taken to avoid inhalation, ingestion, or contact with skin.

2.2. Cell Culture, Protein Expression, and Cell Lysis

1. Cells: 293T (*see* Note 1).
2. Mammalian expression vector encoding Pyo-tagged protein of interest. Typically, we add two tandem copies of the Pyo-tag (EYMPME: 5'-GAG TAT ATG CCC

¹We typically use 293T cells for proteomic studies because they are easily transfectable, exhibit good protein expression, and have a broad endogenous protein expression pattern. In particular, 293T cells are preferable to HeLa cells, in that although HeLa cells are easily transfectable, the endogenous protein expression profile is more restricted and the expression of certain genes has been silenced by methylation in this carcinoma line (e.g., LKB1). We have, however, successfully performed proteomic studies in other cell lines that are difficult to transfect by using adenoviral or retroviral expression systems to transiently or stably express the Pyo-tagged protein.

ATG GAG-3') to the N-terminus of a protein (following an ATG start codon); however, depending on the functional properties of the protein, it may be more desirable to place the tag at the C-terminus.

3. FuGENE 6 (Roche, Indianapolis, IN) for transfection of cells.
4. Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA).
5. Growth Media: DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen).
6. PBS (*see* Subheading 2.1., item 3).
7. Low Salt Triton X-100 lysis buffer: 30 mM Tris (pH 8.0), 75 mM NaCl, 10% glycerol, 1% Triton X-100 containing protease inhibitors (0.15 units/mL aprotinin, 20 μ M leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and phosphatase inhibitors (0.5 mM sodium vanadate, 0.1 μ M calyculin A). Prepare fresh using high purity Triton X-100 (Surfact Amps X-100, supplied as a 10% solution, cat# 28314, Thermo Scientific, Waltham, MA) and prechilled HPLC grade water. Protease and phosphatase inhibitors should be added from concentrated stock solutions immediately prior to use. In particular, PMSF has a very short half-life in aqueous solutions (~20 min).
 - a. 200 mM PMSF: dissolve 0.348 g of PMSF in 10 mL ethanol. Aliquot and store protected from light at -20°C . Note, PMSF will precipitate from solution at -20°C , so briefly warm at 37°C and vortex prior to use.
 - b. 10 mg/mL leupeptin: dissolve 50 mg leupeptin (Roche) in 5 mL of water. Aliquot and store at -20°C .
 - c. 100 mM sodium vanadate: for 10 mL dissolve 183.9 mg sodium vanadate in 10 mL water and adjust to pH 10 (yellow in color). Boil until solution turns colorless then cool to room temperature and adjust the pH to 9. Repeat boil/cool/pH step until solution remains at pH 9. Make 1 mL aliquots and store at -20°C or if in frequent use, store at 4°C . Note, if precipitate is observed after thawing, warm at 37°C and vortex until solution is clear.
 - d. 20 μ M calyculin A: resuspend 10 μ g calyculin A (Cell Signaling Technology, Danvers, MA) in 500 μ L of 50% ethanol/50% water. Store at 4°C .

2.3. Affinity Purification of Scaffold Complexes for Mass Spectrometry Analysis

The following materials and solutions should be designated for use exclusively in proteomic analysis and should always be handled with gloved hands to prevent keratin contamination (*see* Note 2).

- 1 Pyro affinity resin (*from* Subheading 2.1.). Alternatively, Pyro/Glu-Glu Affinity Matrix (Cat # AFC-115P, Covance Inc., Princeton, NJ).
- 2 Low Salt Triton X-100 lysis buffer (*see* Subheading 2.2., item 7)

²MS protein analysis is a highly sensitive technique and the major cause of sample contamination is from keratin found on skin and in dust. We have found that the following precautions can significantly reduce the risk of keratin contamination. First, purchase fresh reagents for use exclusively in mass spectrometry sample preparation and keep them stored away from general lab use. Second, wear double gloves at all times when working with samples and handling proteomics reagents. Third, rinse all surfaces that may contact the gel with HPLC grade water. These surfaces include the outside of gloved hands, gel electrophoresis apparatus, staining boxes, razor blade, and scalpels.

- 3 HPLC grade water (CHROMASOLV for HPLC cat# 270733, Sigma).
- 4 2X gel sample buffer: prepared by diluting 5X sample buffer (250 mM Tris (pH 6.8), 30% Glycerol, 10% sodium dodecyl-sulfate (SDS), 500 mM dithiothreitol (DTT), 0.2% bromophenol blue) with water. 5X and 2X are stored at -20°C .
- 8 4–20% Tris-glycine polyacrylamide minigel, 1.0 mm x 10 wells (Invitrogen) (*see* Note 3).
- 9 SDS-polyacrylamide gel electrophoresis (PAGE) apparatus. Our laboratory has dedicated a gel electrophoresis apparatus for use only in the preparation of samples for mass spectrometry analysis and it is kept stored in a plastic container to reduce keratin contamination from dust. 10. 1X Novex Tris-glycine SDS running buffer: prepared fresh in a clean, well-rinsed graduated cylinder by diluting
- 10 1X Novex Tris-glycine SDS running buffer (Invitrogen) with HPLC grade water.
- 11 Prestained SDS-PAGE protein standards (e.g., BioRad Broad Range, BioRad, Hercules, CA).
- 12 Nalge S700 plastic box (cat# 1917H42, Thomas Scientific, Swedesboro, NJ) for staining and destaining of polyacrylamide gels.
- 13 Fix/Stain solution: Prepare 100 mL by pipetting 70 mL HPLC grade water, 20 mL Methanol, 5 mL Acetic Acid, and 5 mL Coomassie Brilliant Blue R250 (BioRad) directly into the plastic gel box using sterile pipettes (*see* Note 4).
- 14 Destain solution: for 100 mL combine 75 mL HPLC grade water, 20 mL Methanol, and 5 mL Acetic Acid. Use sterile pipettes to prepare directly in the plastic gel box.
- 15 5% Acetic Acid solution in HPLC grade water. Use sterile plastic pipettes to prepare directly in the plastic gel box.
- 16 1.5 mL Eppendorf-style plain microfuge tubes. Do not use tubes that are colored, have Orings, or have been chemically treated. We use Eppendorf Safe-Lock Tubes (Eppendorf part #022363204), which are available from many commercial sources.
- 17 Sterile #10 blade scalpels.

3. Methods

3.1. Generation of Pyo Affinity Resin

To affinity purify Pyo-tagged protein complexes for proteomic analysis, we routinely use protein G sepharose beads in which the Pyo monoclonal antibody has been covalently coupled to the bead matrix. The following is a protocol for the generation of the Pyo-affinity resin; however a Pyo/Glu-Glu affinity resin is commercially available from Covance, Inc.

³Optimal MS analysis requires high-quality gel electrophoresis. It is important to have well focused bands such that the protein is concentrated in the minimum amount of gel. In addition, a gel of 1.0 mm thickness is preferred given that the recovery of peptides after in gel digestion is reduced in thicker gels and that there is greater loss of protein in the staining/destaining process in thinner gels. In our studies, we have had excellent success using commercially available 4–20% gradient polyacrylamide minigels. Moreover, the use of commercially available precast minigels can help reduce keratin contamination.

⁴Excessive stain in a gel slice can interfere with the MS analysis. Diluting the Coomassie Brilliant Blue stain in Fix/Destain solution, allows the gel to be fixed and stained in one step and also permits enough staining to visualize protein bands, while reducing the time required for destaining.

that gives comparable results. This methodology can also be adapted for the coupling of other antibodies to the bead matrix.

1. Wash 500 μ L of protein G sepharose beads with 14 mL PBS in a 15 mL conical tube.
2. Pellet beads by spinning at 228g for 10 min and aspirate wash buffer.
3. Resuspend beads in 1 mL of PBS and transfer to a 50 mL conical tube containing 48 mL Pyo hybridoma tissue culture supernatant. Rinse 15 mL tube with 500 μ L PBS and transfer residual beads to 50 mL tube containing the Pyo supernatant.
4. Incubate for 4 hr at 4°C with rocking.
5. Pellet beads by spinning at 228g for 10 min. Decant the supernatant into a clean tube, leaving 3 mL to resuspend the bead pellet. Transfer resuspended beads to a clean 15 mL conical tube. Rinse 50 mL tube with 3 mL of the decanted supernatant and transfer any residual beads to the 15 mL tube.
6. Pellet beads by spinning at 228g for 5 min (use these conditions for all subsequent bead pelleting steps). Aspirate supernatant and wash beads twice with 12 mL 0.2 M sodium borate [pH 9.0].
7. Pellet beads, aspirate supernatant, and resuspend beads in 14 mL 0.2 M sodium borate [pH 9.0]. Take a 200 μ L aliquot for test sample #1.
8. To crosslink the antibody to beads, add 72.52 mg dimethyl pimelimidate (DMP) (final concentration 20 mM) and incubate for 30 min at room temperature with rocking.
9. Pellet beads, aspirate supernatant, and wash beads once with 0.2 M ethanolamine [pH 8.0].
10. Pellet beads, aspirate supernatant, and resuspend bead pellet in 14 mL 0.2 M ethanolamine [pH 8.0]. Incubate for 2 hr at room temperature on a rocker. Take a 200 μ L aliquot for test sample #2.
11. Pellet beads, aspirate supernatant, and wash beads once with PBS.
12. Pellet beads, aspirate supernatant, and resuspend bead pellet in 1 mL PBS containing 0.01% merthiolate. Store beads at 4°C.
13. Test antibody coupling efficiency.
 - a. Pellet beads in test samples and wash three times with PBS.
 - b. Resuspend bead pellet in 20 μ L 2X SDS-gel sample buffer and analyze by standard SDS-PAGE and Coomassie Brilliant blue staining. If the antibody has been successfully coupled, the IgG heavy chain at 50 kDa will be visible in sample #1, but not in sample #2.

3.2. Cell Culture, Protein Expression, and Cell Lysis

1. Seed 16 10 cm tissue culture dishes with 293T cells at a concentration of 1.0×10^6 cells/dish in Complete Media (see Note 5). Incubate cells overnight at 37°C with 5% CO₂.

⁵Each Pyo-tagged construct should be tested for expression levels in the cell line that will be used for affinity purification of the scaffold complexes in order to determine the number of cells/dishes that will be required for MS sample preparation. In general, we have found that for most well-expressed proteins, lysate from 6-10 10 cm dishes of transfected 293T cells is sufficient for sample preparation.

2. 16–18 hr after plating, take 8 dishes and transfect cells with the plasmid for expression of the Pyo-tagged protein using FuGENE 6 reagent. Transfect 6 μ g of plasmid DNA per 10 cm dish of cells using a 2:1 DNA:FuGENE 6 ratio according to the manufacturer's instructions. After transfection, incubate cells at 37°C with 7% CO₂ for 48 hr. Remaining 8 dishes will serve as the untransfected cell control.
3. 48 hr after transfection, decant media from each dish and carefully wash the cell monolayer with 5 mL ice cold PBS. 293T cells may be very loosely adherent at this stage, and if many of the cells detach from the dish during the wash step, scrape the cells into the PBS using a rubber policeman and proceed as indicated in the next step.
4. Decant wash, add 4 mL PBS to each dish, and scrape cells into the PBS solution using a rubber policeman. Transfer the cell suspension to a pre-chilled 50 mL conical tube, pooling all 8 dishes per condition in one tube. Pellet cells by spinning at 3000g for 10 min at 4°C.
5. Carefully aspirate supernatant and resuspend each cell pellet in 1.5 mL Low Salt Triton X-100 lysis buffer. Transfer each lysate to a clean pre-chilled microfuge tube (designated for proteomics work) and incubate on ice for 20 min, vortexing every 5 min to mix.
6. Centrifuge the samples at 14,000g for 10 min in a 4°C microfuge to pellet unbroken cells, nuclei, and insoluble debris.
7. Transfer lysate to a clean pre-chilled microfuge tube (designated for proteomics work) and centrifuge samples at 14,000g for 5 min in a 4°C microfuge.
8. Transfer lysate to a clean pre-chilled microfuge tube (designated for proteomics work), making sure not to transfer any residual debris.

3.3. Affinity Purification of Scaffold Complexes for Mass Spectrometry

The following is a protocol that we routinely use for the preparation of samples for mass spectrometry (MS) analysis. However, because MS facilities can differ in their requirements for sample preparation and submission, the facility that will be processing the samples should be contacted prior to beginning sample preparation to discuss their specific protocols and requirements. It is also imperative that precautions are taken to avoid keratin contamination during all steps of the protocol.

- 1 Determine the protein concentration of the lysates from **Section 3.2.** using a standard Bradford assay. Typically, we find that the lysate from control untransfected cells has a higher protein concentration than does the lysate from transfected cells expressing the Pyo-tagged protein. If this is the case, normalize the lysates by transferring to a clean prechilled microfuge tube a volume of control untransfected cell lysate that is equivalent in total protein amount to that present in the ~1.5 mL transfected cell lysate. Equalize the volume of the two samples using Low Salt Triton-X100 lysis buffer.
- 2 To each lysate sample, add 25–40 μ L Pyo-affinity resin (from **Section 3.1.**), taking the aliquot directly from the resin pellet. Note, to prevent loss of the Pyo-affinity resin, we do not resuspend the resin by mixing. Spin samples at 3000g for 2 min in microfuge and check pellet to ensure that an equivalent amount of resin was added to each sample. Incubate samples for 4 hr at 4°C with rocking.
- 9 Spin samples at 3000g for 2 min in a microfuge to pellet resin. Using a clean pipet tip for each sample, carefully aspirate the supernatant making sure not to disturb the resin pellet.

- 10 Wash resin pellet, by adding 1 mL of Low Salt Triton X-100 lysis buffer and inverting each tube several times. Note, do not vortex samples as vortexing may cause the sepharose bead matrix to rupture, allowing non-specific proteins to be trapped inside the bead.
- 11 Repeat resin wash step 4 more times.
- 12 On final wash, carefully aspirate the supernatant such that only the resin pellet remains. Immediately resuspend resin pellet in 25 μ L boiling hot 2X gel sample buffer and boil samples for 6 min.
- 13 Briefly spin samples in a microfuge to pellet beads. Load entire sample into one well of a 10 well 4–20% Tris-glycine polyacrylamide minigel. Load protein markers and run gel at 15–20 mAmps.
- 14 After electrophoresis, the following steps put the gel in direct contact with the lab environment and are particularly susceptible to keratin contamination. Extra care should be taken to make sure that hands are double-gloved and that all solutions are handled appropriately. Avoid directly touching the gel, even when wearing gloves, and if the gel needs to be manipulated, do so using a sterile pipet tip.
- 15 Carefully separate the gel plates, allowing the gel to remain on one plate. Cut and remove stacking gel using a clean razor blade. Carefully invert the plate containing the gel over the plastic gel box that contains 100 mL Fix/Stain solution and allow the gel to drop into the Fix/Stain solution. Incubate at room temperature with rocking until protein bands are visualized.
- 16 Decant Fix/Stain solution and destain gel in 100 mL Destain solution at room temperature with rocking. Change solution as needed to destain gel such that the background is clear, but protein bands are still visible.
- 17 After destaining, incubate gel in 100 mL 5% Acetic acid for at least 1 hr (gel can be stored in this buffer). An example of a Coomassie stained gel showing Pyo-affinity purified KSR2 scaffold complexes is shown in Fig. 1.
- 18 To cut protein bands for MS analysis, transfer the gel to a sterile 150 mm tissue culture dish containing 10 mL HPLC grade water and place on a light box. Number eppendorf tubes S1, S2, etc for bands cut from the Pyo-tagged protein sample lane and C1, C2, etc for bands cut from the untransfected control lane. Using a sterile scalpel, cut slices of approximately 5 mm in width moving from the top of the gel to the bottom and transfer the gel slice into the eppendorf tube using the scalpel. Cut slices from both the sample and control lanes, making sure to match the size and position of each control slice with the corresponding sample slice. Typically, we take a picture of the gel before cutting and then label the picture to indicate the corresponding gel slice/tube number. By cutting out the entire lane, one does not limit the identification of potential scaffold binding partners based upon size or band staining. However, if this is cost prohibitive, a smaller number of sample bands can be excised, making sure to cut a corresponding slice from the control lane.
- 19 Submit samples to designated facility for MS analysis or store frozen at -20°C .

3.4 Analysis of Mass Spectrometry Data

Proteins are identified by matching the MS ion spectra of the peptides to SwissProt and NCBI databases using search algorithms like SEQUEST. Therefore, it is important to provide the MS facility with the species of the cell line in which the protein complexes were

derived such that the proper databases can be searched. From the MS analysis, a list will be generated of the peptides derived from a particular protein that is present in each gel slice sample. Determining which of the identified proteins may be a functionally relevant scaffold binding partner requires that a comparison be made between the number of peptides for a particular protein recovered in the experimental sample to that recovered in the control sample. For example, although a sample slice may contain many peptides of protein (such as a highly abundant protein like actin), if an equivalent number of peptides for this protein is also identified in the control slice, this protein would be considered a non-specific interactor. In contrast, if only a few peptides of a protein (in particular, a low abundance protein) were identified in the sample slice, but no peptides were identified in the control slice, this protein may represent a significant scaffold binding partner. Typically, the proteins that we choose for further analysis are those found only in the experimental sample or those significantly enriched in the experimental sample. Functionally relevant binding partners for the KSR scaffolds that fall into the latter category are members of the 14-3-3 family. The 14-3-3s are highly abundant proteins, and although a few peptides derived from these molecules are often found in control samples, a 5 fold greater number of 14-3-3 peptides are found in KSR samples. It is also important to note that the MS analysis will detect proteins associated with the translation and folding of the overexpressed Pyo-tagged scaffold, such as Hsp70, chaperonins, and ribosomal binding proteins, and these interacting proteins are not routinely investigated further.

3.5. Confirmation of Interactions

Binding interactions identified in the proteomics analysis will need to be further validated. Typically, we confirm these interactions by co-immunoprecipitation assays in which the scaffold complex is immunoprecipitated under conditions similar to those described in **Section 3.3.** and the presence of the putative binding partner is detected by immunoblot analysis. Co-immunoprecipitation assays can be conducted using transfect cells or cell lines stably expressing the Pyo-tagged protein, or using cells with endogenous expression of the proteins of interest. Once the interactions have been confirmed, these assays can also be adapted to evaluate the interactions under various signaling conditions (e.g. serum starvation or growth factor treatment) or using mutant proteins to determine the residues/domains required for binding.

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Fig. 1. Coomassie Stained Gel of KSR2 Scaffold Complexes. Pyo-tagged full-length (FL) KSR2 or Pyo-tagged N-terminal (N') or C-terminal (C') domains of KSR2 were expressed in 293T cells. Scaffold complexes were isolated from cell lysates using the Pyo-affinity resin and resolved by electrophoresis on a 4–20% Tris-glycine polyacrylamide gel. Arrows indicate the KSR2 proteins, as well as some of the KSR2 binding partners identified by MS analysis.