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Analyzing FAK and Pyk2 in Early Integrin Signaling Events

Integrins are a family of heterodimeric α/β transmembrane receptors that bind to extracellular matrix proteins (ECM) proteins, and the signals generated by activated integrins regulate cell motility (Schwartz, 2001; Hynes, 2002). As integrins do not possess intrinsic signaling activity, integrin-stimulated signaling events promoting cell motility are initiated by integrin-associated kinases, e.g., focal adhesion kinase (FAK). FAK is one of several intracellular protein-tyrosine kinases (including the Src family, Pyk2, c-Abl, and Syk) that are activated by cell adhesion to ECM proteins such as fibronectin, collagen, or vitronectin. Integrin activation of FAK promotes increased FAK tyrosine phosphorylation and leads to the formation of an FAK-Src signaling complex (Schlaepfer et al., 1999; Schlaepfer and Mitra, 2004). Fibroblasts lacking FAK spread poorly and display migration defects in response to integrin stimuli (Ilic et al., 1995). Although the FAK-related kinase Pyk2 is expressed in FAK-null fibroblasts (FAK^{-/-}), Pyk2 is not as effective in promoting FAK^{-/-} cell motility as the FAK-Src signaling complex (Sieg et al., 1998). This unit describes methods for culturing FAK^{+/+} and FAK^{-/-} mouse embryo fibroblasts (MEFs; see Support Protocol 1) and stimulating these cells by replating onto fibronectin-coated dishes (see Basic Protocol 1). It also contains support protocols for cell starvation (see Support Protocol 2), preparation of protein cell lysates (see Support Protocol 3), and conditions and antibodies available for immunoprecipitation of FAK, Pyk2, and c-Src (see Support Protocol 4).

Cell replating onto fibronectin activates FAK, and procedures are described for analyzing FAK-associated autophosphorylation activity (see Basic Protocol 2) with alternate protocols describing assays for measuring FAK-Pyk2 phosphorylation of a peptide substrate (see Alternate Protocol 1) and changes in c-Src-associated *in vitro* kinase activity (see Alternate Protocol 2). These catalytic measurement assays are complemented by methods using antibodies available for evaluating activity changes in FAK, Pyk2, or c-Src using immunoblotting techniques with phospho-specific antibodies (see Basic Protocol 3). In addition, the biochemical analyses are complemented by cell biological methods to evaluate integrin-stimulated haptotaxis migration (see Basic Protocol 4) and time-lapse imaging to monitor wound closure motility in culture (see Basic Protocol 5). In these cell motility assays, transient plasmid expression vector transfection can be used to overexpress various proteins to test their effects on cell motility (Alternate Protocol 3). Finally, protocols are provided for visualizing activated FAK, Pyk2, and c-Src in cells plated onto extracellular matrix proteins (see Basic Protocol 6) with support methods for staining filamentous actin in cells (see Alternate Protocol 4), an easy means to visualize changes in cell shape and indirectly localize integrin-containing focal adhesions that are found at the ends of actin stress fibers.

NOTE: All culture incubations should be performed in a humidified 37°C, 10% CO₂ incubator unless otherwise specified. Some media, e.g., DMEM, require higher levels of CO₂ to maintain pH 7.4.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

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Signal
Transduction:
Protein
Phosphorylation

14.7.1

Supplement 30

REPLATING ASSAYS FOR SIGNALING STUDIES

Although anti-integrin antibodies can be used to facilitate integrin clustering, one of the strongest activators of integrin signaling is replating or passaging cells onto ECM-coated culture dishes. In the span of 1 hr, cells will rapidly bind to the ECM protein provided and will undergo cell spreading and morphology changes. Fibronectin binds to variety of α/β integrin pairs expressed on cells and is the strongest activator of FAK. Other ECM proteins can be used in replating assays, although the effective concentrations needed to stimulate integrin signaling are varied and dependent on cell type. This protocol describes the optimal conditions for replating FAK^{+/+} and FAK^{-/-} cells on fibronectin to achieve maximal FAK or Pyk2-Src activation, respectively. It also provides a method for collecting protein lysates from these cells for further signaling studies. For comparative purposes, cell lysates are collected from adherent serum-starved cells, cells that have been held in suspension, cells replated onto fibronectin, and cells that have been replated onto a positively-charged ligand such as poly-L-lysine where rapid cell adhesion does not depend on integrin engagement.

Materials

Fibronectin (from bovine plasma; Sigma-Aldrich)
Poly-L-lysine (mol. wt. 70,000 to 150,000 or 150,000 to 300,000; Sigma-Aldrich)
Replating and migration medium (see recipe)
FAK^{+/+} and FAK^{-/-} MEFs, serum-starved (Support Protocol 2)
Phosphate buffered saline (PBS; APPENDIX 2A)
Trypsin/EDTA: 0.25% (w/v) trypsin/1 mM EDTA (Invitrogen)
Trypsin inhibitor solution (see recipe)
10-cm plastic tissue culture plates (Falcon)
4°C incubator
15-ml centrifuge tubes (Corning)
Tabletop centrifuge
10-ml transfer pipets, sterile
50-ml conical centrifuge tubes (Corning)
Light microscope

Coat plates and prepare cells

- 1a. *To prepare fibronectin plates:* Dilute fibronectin to 10 $\mu\text{g/ml}$ in PBS and distribute 5 ml per 10-cm plastic tissue culture plate. Incubate at 4°C overnight.
- 1b. *To prepare poly-L-lysine plates:* Dilute poly-L-lysine to 50 $\mu\text{g/ml}$ in PBS and distribute 5 ml per 10-cm plastic tissue culture plate. Incubate at 4°C overnight.
2. Prior to preparing cells, aspirate fibronectin or poly-L-lysine solutions, add 3 ml of replating and migration medium, aspirate, and place dishes at 37°C.

The number of cell plates prepared depends on the number of experimental points to be evaluated. For example, to perform a time course of FAK activation, it is recommended that fibronectin and poly-L-lysine replating time points of 20, 40, 60, and 180 min be performed (see Support Protocol 3).

3. Reserve one plate of serum-starved cells for a control. Wash serum-starved cells with PBS by adding 10 ml PBS and aspirating it off with a transfer pipet. Add 2.5 ml trypsin/EDTA per dish. Tap side of plates to facilitate cell release.
4. Add 5 ml trypsin inhibitor solution warmed to 37°C. Transfer cells to a 15-ml centrifuge tube. Centrifuge 3 min at $750 \times g$, 21°C, in a tabletop centrifuge and aspirate supernatant.

Replate cells

5. Resuspend cells in 37°C replating and migration medium to 2×10^5 cells/ml. Keep cells in suspension at least 30 min and rotate tube gently at least every 5 min to keep cells from clumping, settling, and sticking to the plastic.

It is convenient to pool all suspended cells into 50-ml conical centrifuge tubes such that subsequent cell distribution in replating assays is consistent. FAK^{-/-} cells will sometimes clump together in suspension and forced pipetting to break apart clumps is recommended prior to replating. Keep additional cells in suspension during replating.

6. Add 5 ml cell suspension to a 10-cm plate pre-coated with fibronectin or poly-L-lysine (from step 3) and incubate at 37°C.
7. Periodically visualize replated cells under a microscope for changes in cell attachment and spreading.

Maximal FAK activation occurs when cells have bound to fibronectin and are undergoing rapid cell spreading (between 20 and 60 min). Cells on poly-L-lysine will adhere, but will be very slow to spread.

8. Proceed with Support Protocol 3 to prepare cell lysates of control cells (adherent serum-starved cells), experimental cells (replated on fibronectin or poly-L-lysine), and unplated serum-starved cells in suspension (45 min post replating).

The unplated serum-starved cells in suspension and serum-starved experimental cells will be very important in correctly evaluating changes in FAK activation in immunoblotting (Basic Protocol 3) and in vitro kinase assays (Basic Protocol 2).

GROWTH OF FAK^{+/+} AND FAK^{-/-} FIBROBLASTS

FAK-null MEFs were derived from an E8.0-day-old mouse embryo that had null mutations in genes for FAK and p53 (Ilic et al., 1995). Therefore, the cells are genetically FAK^{-/-} p53^{-/-}. Control FAK^{+/+} cells were derived in 1995 from an E8.0-day-old mouse embryo that had null mutations in p53 but not in FAK. Therefore, the cells are genetically FAK^{+/+} p53^{-/-}. Both cell lines were derived from embryos of littermates obtained by crossing FAK^{+/-} p53^{-/-} female and FAK^{+/-} p53^{-/-} male mice.

FAK^{+/+} and FAK^{-/-} MEFs are grown on gelatin precoated plates because this was used during cell outgrowth from embryos. Although the fibroblasts are p53-null, the cells will become senescent (exhibited by an increase in cell size and the lack of cell proliferation) at high passage numbers.

Materials

Gelatin
Phosphate buffered saline (PBS; APPENDIX 2A)
FAK^{+/+} mouse embryo fibroblasts (ATCC CRL-2645) FAK^{-/-} mouse embryo fibroblasts (ATCC CRL-2644): grown on plates as per ATCC product sheet
Trypsin/EDTA: 0.25% (w/v) trypsin/1 mM EDTA (Invitrogen), 37°C
Cell growth medium (see recipe), warmed to 37°C
42°C water bath
0.22- μ m GP Express Plus filter membrane (Millipore)
10-cm plastic tissue culture plates (Falcon)
10-ml transfer pipets, sterile
Light microscope
15-ml conical centrifuge tubes (Corning)

SUPPORT PROTOCOL 1

Signal
Transduction:
Protein
Phosphorylation

14.7.3

Prepare gelatin-coated plates

1. Dissolve 0.5 g gelatin in 500 ml PBS to make 0.1% (w/v) solution.
2. Heat in 42°C water bath to dissolve gelatin.
3. Sterilize by passing through a Millipore 0.22- μ m GP Express Plus filter membrane. Store up to 2 weeks at 4°C.
4. Coat 10-cm plastic tissue culture plates with 5 ml sterile 0.1% gelatin solution and incubate 1 hr at 37°C
5. Aspirate gelatin solution. Do not wash plates.

Trypsinize cells

6. Aspirate growth medium from a 10-cm plate of FAK^{-/-} or FAK^{+/+} mouse embryo fibroblasts.
7. Wash cells with 5 ml PBS.
8. Add 2.5 ml warm trypsin/EDTA per plate. Tap side of plates to facilitate cell release.
9. Incubate 2 to 3 min. Verify cell detachment under a microscope.

FAK^{-/-} cells adhere to the culture plates more firmly than FAK^{+/+} cells and will take longer to be released by trypsin/EDTA treatment. Senescent FAK^{-/-} cells exhibit very strong adhesion to gelatin-coated dishes.

10. Inactivate trypsin/EDTA by adding 5 ml cell growth medium.

Replate cells

11. Wash cells off plates by pipetting, and transfer them into 15-ml conical centrifuge tubes.
12. Centrifuge cells in a tabletop centrifuge 3 min at 750 \times g, 21°C.
13. Aspirate supernatant and discard, retaining the pellet.
14. Resuspend cells in fresh growth medium, count (see UNIT 1.1), and add growth medium to obtain the desired density. Add to gelatin-coated plates containing cell growth medium.

Total volume is 8 to 10 ml medium per 10-cm plate.

Consistent subcultivation is very important as cells that remain confluent for more than 24 hr exhibit alterations in cell morphology and motility properties. In routine passage, FAK^{-/-} cells may proliferate faster than FAK^{+/+} cells, so cell counting and normalization of cell numbers is required for equivalent experimental analyses.

Both FAK^{-/-} and FAK^{+/+} cells contain neomycin resistance genes and G418 (neomycin) selection can be applied (500 μ g/ml), although this is not absolutely required.

15. Passage the cells when they are confluent.

Split cells 1:10 to 1:15 every 3 days. Do not allow cells to become overly confluent, as this can result in a premature senescent phenotype.

SERUM STARVATION OF CELLS

For the stimulation and analysis of FAK activity and phosphorylation (Basic Protocols 1, 2, and 3) and assays that involve cell replating onto extracellular matrix (ECM) proteins (Basic Protocols 4, 5, and 6), it is important to start with cells that are adherent and in a quiescent or nonproliferative state. This is accomplished by lowering the percent of serum to 0.5% in the growth medium.

SUPPORT PROTOCOL 2

Analyzing FAK and Pyk2 in Early Integrin Signaling

14.7.4

Additional Materials (also see Support Protocol 1)

Starvation medium: prepared by making cell growth medium (see recipe) with FBS reduced to 0.5%.

1. Passage cells (see Support Protocol 1) by plating 1×10^6 cells onto gelatin-coated 10-cm dishes in growth medium.

This is a subconfluent density, and it is important that cells do not reach confluency prior to initiation of cell motility analyses.

2. After 24 hr, wash cells with PBS.

3. Add 8 ml of starvation medium and incubate 16 to 24 hr at 37°C. Use cells immediately after starvation.

Greater than 95% of FAK^{-/-} and FAK^{+/+} cells should remain adherent under serum-starvation conditions.

PREPARATION OF CELL LYSATES

Activated FAK is associated with the cytoskeleton-rich fraction of cells that is not easily solubilized with low-detergent-containing buffers. The following lysate preclearing steps are used as the first step for immunoprecipitation, blotting, and in vitro kinase assays. All procedures are to be performed at 4°C.

Materials

Experimentally treated, control adherent, and control suspended cells (Basic Protocol 1)

Phosphate-buffered saline (PBS; APPENDIX 2A), 4°C

RIPA cell lysis buffer (see recipe), 4°C

50% (w/v) Sephadex G-100 (Sigma G100-120) slurry in PBS (APPENDIX 2A)

Cell scraper (Corning)

Refrigerated centrifuge

1.5-ml microcentrifuge tubes

1-ml disposable syringes

21-G Luer-Lok needles

Tube rotation device (e.g., Labquake, Barnstadt-Thermolyne)

Microcentrifuge

-80°C freezer (optional)

1. Wash experimentally treated and adherent control cells with 10 ml cold PBS.
2. At 45 min after replating the experimental cells (see Basic Protocol 1), centrifuge control suspended cells 3 min at $750 \times g$, 21°C. Resuspend cells in PBS and repeat centrifugation.
3. Add 750 μ l cold RIPA cell lysis buffer to each 10-cm plate (or 1×10^6 suspended cells).
4. Scrape adherent cells using cell scraper and transfer to a 1.5-ml microcentrifuge tube.
5. Using a 1-ml disposable syringe and 21-G Luer-Lok needle, pull cell lysate into syringe and shear cellular DNA by extrusion. Repeat at least five times.

Be careful using the sharp needle and do not use too much force as this will result in excessive bubbling of the lysate.

**SUPPORT
PROTOCOL 3**

**Signal
Transduction:
Protein
Phosphorylation**

14.7.5

6. Add 100 μ l 50% Sephadex G-100 slurry in PBS to each tube. Rotate tubes 10 min at 4°C.

Sephadex G-100 beads will reduce DNA contamination and non-specific protein binding for subsequent immunoprecipitation analyses.

7. Centrifuge 10 min at 16,000 \times g, 4°C.
8. Transfer supernatant (cleared total cell lysate) into new 1.5-ml microcentrifuge tubes.
If the bead pellet is distributed along the sidewall of the microcentrifuge tubes, rotate tube direction 180° in centrifuge rotor and centrifuge an additional 5 min.
9. Store cleared lysate indefinitely at -80°C or proceed with subsequent analyses.

IMMUNOPRECIPITATION OF FAK, Pyk2, AND c-Src

FAK is expressed at high levels in FAK+/+ fibroblasts. The FAK-related kinase, Pyk2, is expressed at low levels in FAK+/+ fibroblasts and at high levels in FAK-/- fibroblasts. Upon replating on fibronectin, both FAK and Pyk2 will form transient signaling complexes with the c-Src protein-tyrosine kinase. Replating of cells on fibronectin can also lead to the activation of c-Src through dephosphorylation of Tyr-527 located in the C-terminal domain of the regulatory region. This protocol describes standard immunoprecipitation methods used to isolate FAK, Pyk2, or multi-protein complexes with c-Src.

Materials

Primary antibody (see Table 14.7.1 and Table 14.7.2)
Lysate of treated or control cells (Support Protocol 3)
50% (w/v) protein A-agarose beads (fast flow immobilized protein A; Repligen, <http://www.repligen.com>) in PBS (APPENDIX 2A)
50% (w/v) protein G-Plus-agarose beads (Calbiochem) in PBS (APPENDIX 2A)
Triton lysis buffer (see recipe), 4°C
HNTG buffer (see recipe), 4°C
1.5-ml microcentrifuge tubes
Tube rotation device (e.g., Labquake, Barnstadt-Thermolyne)
Centrifuge
Transfer pipets

1. Add 1 to 2 μ g of primary antibody to 0.75 ml lysate of treated or control cells in a microcentrifuge tube.

If using cells from a frozen lysate, thaw at 37°C then maintain at 4°C.

Use a FAK or Pyk2 antibody for subsequent analysis of FAK/Pyk2 kinase activity or phosphorylation. (Basic Protocol 2 or Alternate Protocol 1).

Use an Src antibody for subsequent Src-associated analysis (Alternate Protocol 2).

2. Rotate from 3 to 14 hr at 4°C.
3. Add 30 μ l of protein A- or protein G-bead slurry and rotate 60 min at 4°C.
Protein A beads are used to capture rabbit polyclonal antibodies and protein G beads are used to bind goat polyclonal and mouse monoclonal antibodies (Table 14.7.1 and Table 7.2.1).
4. Centrifuge beads 1 min at 1000 \times g, 4°C. Remove supernatant with a transfer pipet.
5. Add 800 μ l Triton lysis buffer to beads, invert tube to suspend the bead pellet, and centrifuge 1 min at 1000 \times g, 4°C. Remove supernatant with a transfer pipet and repeat step two more times.

6. Add 800 μ l of HNTG buffer, invert the tube to suspend the bead pellet, and centrifuge 1 min at 1000 \times g, 4°C. Remove supernatant with a transfer pipet and repeat step two more times.

From this point, the immunoprecipitated samples can be analyzed in either the in vitro kinase assay (Basic Protocol 2) or resolved by SDS-PAGE (UNIT 6.1) and processed by immunoblotting (UNIT 6.2 and Basic Protocol 3).

IN VITRO KINASE ASSAY

Integrin binding to fibronectin leads to rapid cell spreading, and FAK, Pyk2, and c-Src tyrosine kinases become activated. The process can be characterized through changes associated with in vitro kinase activity. Assays are designed to detect auto-phosphorylation or the intramolecular phosphorylation of an added substrate such as a synthetic poly (Glu:Tyr) peptide for FAK and Pyk2, or the phosphorylation of a recombinant fragment of the FAK C-terminal domain by c-Src.

Materials

- Immunoprecipitated samples (Support Protocol 4, step 6)
- FAK-Pyk2 kinase buffer (see recipe), 4°C
- Src kinase buffer (see recipe), 4°C
- 10 μ Ci/ μ l [γ ³²P]ATP (>3,000 Ci/mmol; PerkinElmer)
- Magnesium/ATP cocktail (Upstate Biotechnology or see recipe)
- 2 \times Laemmli SDS buffer (see recipe)
- Coomassie blue stain (see recipe)
- Molecular weight marker (Precision Plus, Bio-Rad)
- Destaining solution (see recipe)
- Microcentrifuge
- 1.5-ml microcentrifuge tubes
- 32°C water bath
- Plexiglas shielding
- Gloves
- Geiger counter
- Plexiglas box
- Micro tube cap locks (RPI 145063; <http://www.rpicorp.com>)
- Whatman 3MM filter paper
- Gel dryer
- Immobilon PVDF membrane (Millipore IPFL 000-10)
- Additional reagents and equipment for SDS-PAGE (UNIT 6.1), autoradiography (UNIT 6.3), and transfer of proteins to membranes for immunoblotting (UNIT 6.2)

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following guidelines provided by the local radiation safety officer (also see APPENDIX 1A).

1. Wash the immunoprecipitated samples (Support Protocol 4, step 6) with 800 μ l cold FAK-Pyk2 kinase buffer two times, centrifuging 2 min at 16,000 \times g, 4°C, each time.
2. Aspirate supernatant, but leave a small amount above pellet (~20 to 30 μ l).
3. For FAK-Pyk2 autophosphorylation assays, add 2.5 μ l of 10 μ Ci/ μ l [γ ³²P] ATP and 5 μ l magnesium/ATP cocktail to the immune complexes. Slightly flick tubes to mix and place in 32°C water bath for 15 min.

BASIC PROTOCOL 2

Signal
Transduction:
Protein
Phosphorylation

14.7.7

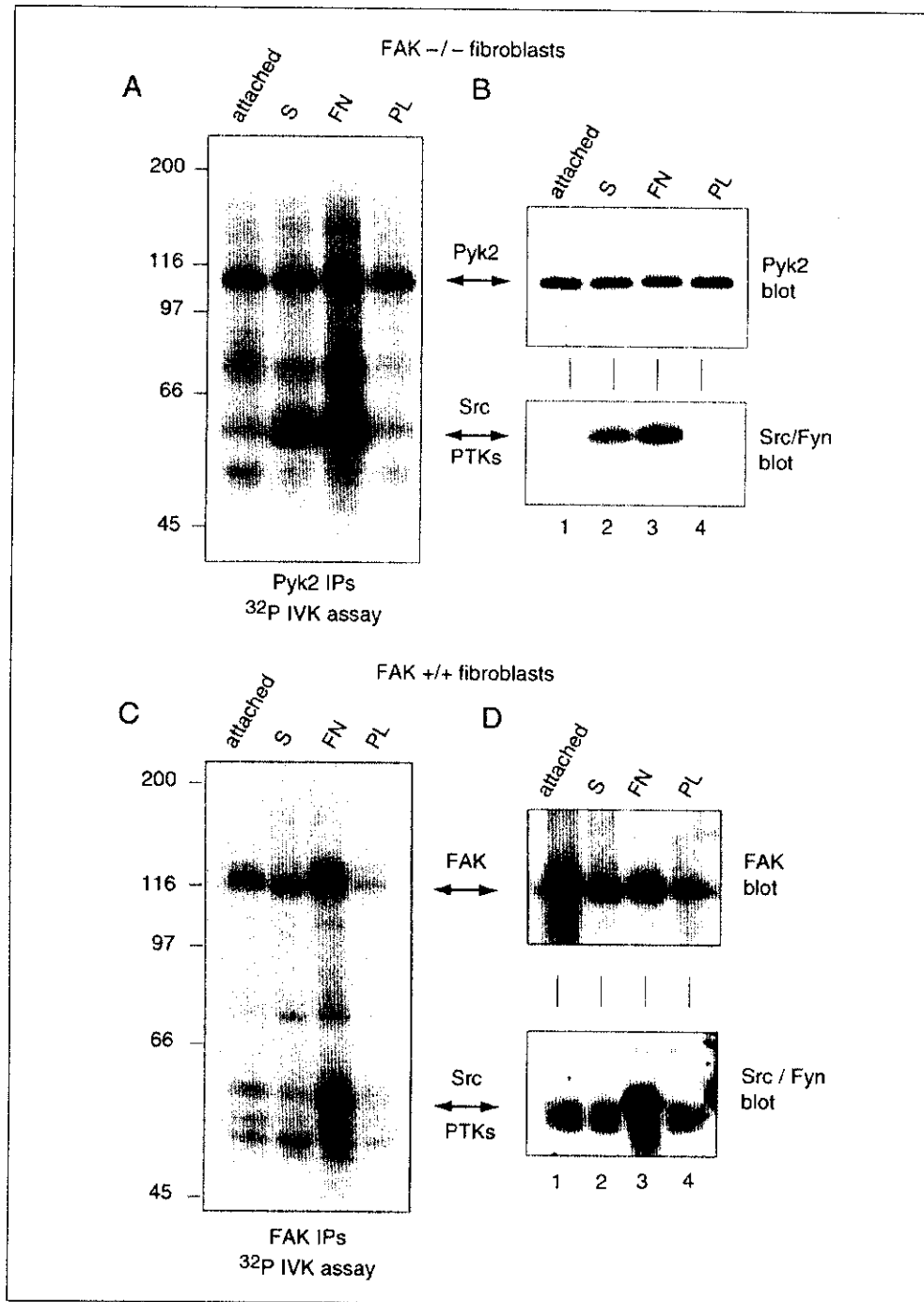


Figure 14.7.1 Measurements of Pyk2 of FAK-associated in vitro kinase activity. Lysates from serum-starved (attached), suspended (S) fibronectin-plated (FN), and poly-L-lysine-plated (PL) FAK^{-/-} cells (A and B) or FAK^{+/+} cells (C and D) were prepared and divided into equal aliquots for either Pyk2 immunoprecipitates (IPs) or FAK IPs, respectively. (A) Pyk2 IPs were labeled by the addition of [γ -³²P]ATP in an in vitro kinase (IVK) assay. ³²P-labeled proteins were transferred to membranes and visualized by autoradiography. (B) The same membrane shown in (A) was cut and analyzed by either anti-Pyk2 or anti-Src family PTK blotting. (C) FAK IPs were labeled by the addition of [γ -³²P]ATP in an IVK assay and the same membrane (D) was cut and analyzed by either anti-FAK or anti-Src family protein-tyrosine kinase (PTK) immunoblotting. Molecular weight standards are indicated in kDa to the left. (previously published in the *EMBO Journal*; Sieg et al., 1998)

CAUTION: Place Plexiglas shielding around gel area to prevent radiation exposure. Remember to check gloves and work areas with a Geiger counter after the kinase assay is done. Use a Plexiglas box for moving radioactive materials within the laboratory and dispose of radioactive waste into designated storage according to institutional requirements.

4. Add 50 μ l 2 \times Laemmli SDS buffer to stop kinase reactions. Secure 1.5-ml tubes with micro tube cap locks.
5. Boil samples for 3 min at 100°C.
6. Microcentrifuge agarose beads 2 min at 16,000 \times g, 21°C. Load supernatants and molecular weight marker onto a 7.5% SDS-PAGE gel (see UNIT 6.1).

For autoradiography

- 7a. After gel is run (~4 hr), cut one top corner to mark orientation. Place gel in Coomassie blue stain for 1 hr and then destain gel for 6 to 12 hr in multiple changes of destaining solution on a shaking platform. Wash a final time in water.
- 8a. Place gel onto Whatman 3MM filter paper and dry using a gel dryer.
- 9a. Expose gel to film or phosphor-imager screen (UNIT 6.3) to quantify ³²P incorporated into FAK (~116 kDa), Pyk2 (~110 kDa) or associated c-Src (~60 kDa).

See Fig. 14.7.1 A and C.

For autoradiography and subsequent immunoblotting

- 7b. Transfer the gel to a PVDF membrane via semidry electrophoretic transfer (see UNIT 6.2).
- 8b. Stain min with Coomassie blue stain. Destain 10 min in destaining solution and wash a final time in water.
- 9b. Place membrane between protective plastic sheets and expose gel to film or phosphor-imager screen to quantify ³²P incorporated into FAK (~116 kDa), Pyk2 (~110 kDa) or associated c-Src (~60 kDa). See UNIT 6.3 for more information.

In this manner, subsequent immunoblotting (see Basic Protocol 3) can be performed on the kinase reactions to verify the amount of FAK or Pyk2 in the immune complex or the amount of c-Src associated with the activated FAK and/or Pyk2 complex reaction (see Fig. 14.7.1B and D).

FAK-PYK2 POLY GLU:TYR PHOSPHORYLATION

This procedure is used to measure the autophosphorylation of FAK/Pyk2 (as in Basic Protocol 2) combined with the transphosphorylation of an added generic substrate peptide.

Additional Materials (also see Basic Protocol 2)

- Immunoprecipitated samples (see Support Protocol 4, step 6)
- 10 mg/ml poly(Glu:Tyr; 4:1) mol. wt. 20,000 to 50,000; sodium salt (Sigma-Aldrich) prepared in PBS and stored up to 2 years at -20°C in 1-ml aliquots
- Magnesium/ATP cocktail (Upstate Biotechnology or see recipe)
- 10 μ Ci/ μ l [γ ³²P]ATP (>3,000 Ci/mmol; PerkinElmer)
- FAK-Pyk2 kinase buffer
- 0.75% phosphoric acid
- 100% acetone
- Scintillation fluid (Sigma)
- 2 \times 2-cm Whatman 3MM filter paper squares

ALTERNATE PROTOCOL 1

**Signal
Transduction:
Protein
Phosphorylation**

14.7.9

Conical 50-ml centrifuge tube

Scintillation vials

Scintillation counter

Additional reagents and equipment for preparing immunoprecipitated samples
(Support Protocol 4)

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1A*).

1. Wash the immunoprecipitated samples (from Support Protocol 4, step 6) with 800 μ l cold FAK-Pyk2 kinase buffer two times, centrifuging 2 min at $16,000 \times g$, 4°C , each time.
2. Remove supernatant, but leave a small amount above pellet (~ 20 to 30μ l).
3. Add 5 μ l 10 mg/ml poly(Glu:Tyr), 5 μ l magnesium/ATP cocktail, and 2.5 μ l of 10 $\mu\text{Ci}/\mu\text{l}$ [γ - ^{32}P]ATP (25 μCi) to the immunoprecipitated samples. Slightly flick tubes to mix and place in 32°C water bath for 15 min. Include a negative control for the phosphorylation assay, substituting 35 μ l kinase buffer for the immunoprecipitated samples.

CAUTION: Place Plexiglas shielding around gel area to prevent radiation exposure. Remember to check gloves and work areas with a Geiger counter after the kinase assay is done. Use a Plexiglas box for moving radioactive materials within the laboratory and dispose of radioactive waste into designated storage according to institutional requirements.

The negative control will measure the level of background binding of [^{32}P ATP] to the assay squares.

The total volume of immune complex reaction is $\sim 50 \mu$ l yielding a final concentration of 50 μM ATP from the magnesium/ATP cocktail and 1 mg/ml poly Glu:Tyr.

4. Transfer a 10- μ l aliquot onto the center of a labeled (with a #2 pencil) 2×2 -cm Whatman filter paper square.

There should be enough volume for triplicates for each immune complex reaction.

5. Allow the radiolabeled substrate to bind to filter paper for at least 1 min. Transfer filter papers to a 50-ml conical tube containing 40 ml 0.75% phosphoric acid. Gently invert tube to wash the assay squares for 5 min. Repeat three times and discard liquid as radioactive waste.

Ten or more assays squares can be washed per 50-ml tube.

6. Wash assay squares once with 40 ml of 100% acetone for 5 min. Discard liquid waste and transfer assay squares to scintillation vial. Add scintillation fluid and evaluate using a scintillation counter.

For information on creating a standard curve, see <http://www.perkinelmer.com>.

ALTERNATE PROTOCOL 2

Analyzing FAK and Pyk2 in Early Integrin Signaling

14.7.10

MEASUREMENTS OF Src-ASSOCIATED KINASE ACTIVITY

As c-Src contains both stimulatory (Tyr-416) and inhibitory (Tyr-527) phosphorylation sites that act to regulate catalytic activity, measurements to analyze changes in c-Src-associated kinase activity are performed by c-Src immune complex phosphorylation of an FAK C-terminal domain fragment that contains the known c-Src phosphorylation sites, FAK Tyr-861 and FAK Tyr-925.

Additional Materials (also see *Basic Protocol 2*)

- 10 mg/ml GST-FAK 853-1052 (Schlaepfer lab)
- Enolase (Sigma-Aldrich E-0379), optional
- 50 mM HCl, optional
- 1 M PIPES, pH 7, optional
- 30°C water bath, optional

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1A*).

1. Wash the immunoprecipitated samples (from step 6 in Support Protocol 4) with 800 μ l cold FAK-Pyk2 kinase buffer two times, centrifuging 2 min at 16,000 \times g, 4°C, each time.
2. Remove supernatant, but leave a small amount above pellet (~20 to 30 μ l)
3. Add 5 μ l of 10 mg/ml GST-FAK 853-1052, 5 μ l magnesium/ATP cocktail, and 2.5 μ l of 10 μ Ci/ μ l [γ ³²P]ATP (25 μ Ci) to the immunoprecipitated samples. Slightly flick tubes to mix and place in 32°C water bath for 15 min.

CAUTION: Place Plexiglas shielding around gel area to prevent radiation exposure. Remember to check gloves and work areas with a Geiger counter after the kinase assay is done. Use a Plexiglas box for moving radioactive materials within the laboratory and dispose of radioactive waste into designated storage according to institutional requirements.

The total volume of immune complex reaction is ~50 μ l yielding a final concentration of 50 μ M ATP from the magnesium/ATP cocktail and 1 mg/ml GST-FAK 853-1052.

Alternatively, acid-denatured enolase may be used as a substrate. Enolase must be freshly prepared: Add 10 μ l of enolase (1 mg/ml) to 2 μ l of 50 mM HCl and incubate 10 min at 30°C. Neutralize with 2 μ l 1 M PIPES, pH 7.0, and use 2 μ l acid-denatured enolase per kinase reaction.

4. Proceed with Basic Protocol 2, steps 4 through 9 to perform SDS-PAGE and autoradiography or autoradiography and immunoblotting.

IMMUNOBLOTTING WITH FAK/PYK2 PHOSPHO-SPECIFIC ANTIBODIES

While *in vitro* kinase assays will reflect changes in catalytic activity, immunoblotting with FAK/PYK2 phospho-specific antibodies can be used to assess specific phosphorylation site modification(s) within the kinases. For example, FAK and Pyk2 first become phosphorylated at their autophosphorylation sites Y397 and Y402 respectively. Upon phosphorylation and binding of c-Src in an active signaling complex, c-Src can phosphorylate other sites on FAK and Pyk2. Additionally, there are several serine-threonine sites on FAK that can be phosphorylated by other cellular kinases. Finally, phospho-specific antibodies have been developed to indirectly measure the activation state of c-Src.

Materials

- 2 \times Laemmli SDS buffer (see recipe)
- Immunoprecipitated samples (Support Protocol 4, step 6)
- 100% and 20% methanol
- Transfer buffer (see *UNIT 6.2*)
- EZBlue gel staining reagent (Sigma)
- BSA blocking buffer (see recipe)
- Primary antibody (see Tables 14.7.1 and 14.7.2)

BASIC PROTOCOL 3

Signal
Transduction:
Protein
Phosphorylation

14.7.11

Tris-buffered saline with Tween (TBST, see recipe)
Secondary antibody: Horseradish peroxidase (HRP)-conjugated appropriate species (Pierce)
Enhanced chemiluminescence (ECL) western detection solution (Amersham RPN 2132)
Western blot stripping buffer (see recipe)
Immobilon PVDF membrane (Millipore)
Rotating shaker
Additional reagents and equipment for gel electrophoresis (UNIT 6.1) and electrophoretic transfer (UNIT 6.2)

Perform gel electrophoresis

1. Add 50 μ l of 2 \times Laemmli SDS buffer to the immunoprecipitated samples from Support Protocol 4, step 6 and boil samples 3 min at 100°C.
2. Centrifuge 2 min at 16,000 \times g, 21°C. Using the supernatants perform SDS-PAGE (UNIT 6.1), including molecular size markers.

Transfer proteins to PVDF membrane

3. Transfer proteins to a PVDF membrane using an electrophoretic transfer unit (UNIT 6.2).

PVDF membrane should be prepared by soaking in 100% methanol followed by the appropriate transfer buffer before use.

4. After transfer is complete, stain protein bands with EZBlue gel staining reagent. Rinse membrane in water and destain in 20% methanol until background staining disappears.
5. Photocopy membrane for record keeping purposes and to note positions of protein size markers.

Probe membrane

6. Place membrane in BSA blocking buffer for 1 hr at room temperature.
7. Remove blocking buffer and incubate membranes with primary antibodies 3 hr at room temperature or overnight at 4°C.

A 1:1000 dilution (~0.5 μ g/ml in BSA blocking buffer) is a good starting point for using an antibody for the first time.

8. Wash membranes by rotational shaking with TBST for 10 min. Repeat three times using fresh TBST.
9. Dilute secondary antibody 1:5000 to 1:10,000 in TBST (e.g., 5 μ l in 15 ml) and incubate membranes with the antibody 1 hr at room temperature.

HRP-conjugated secondary antibodies commonly used are: goat anti-mouse IgG, Protein A, donkey anti-rabbit IgG, and mouse anti-goat IgG.

10. Wash membranes by rotational shaking with TBST for 10 min. Repeat three times using fresh TBST.
11. Incubate in 5 ml ECL solution for 5 min at 21°C. Expose to film 30 sec to 10 min to achieve optimal exposure.

Although many phospho-specific antibodies can be used to probe FAK, Pyk2, or c-Src activation states in whole cell lysates, multiple immunoreactive bands detected can complicate the interpretation of results. This is not a problem when analyzing FAK, Pyk2, or c-Src by immunoprecipitation.

Table 14.7.1 Commercially Available Antibodies to FAK, Pyk2, and c-Src^a

Antibody	Company	Source	Application	Catalog number
FAK, clone 4.47	Upstate	mouse (mAb)	WB, IP, IC, IH	05-537
FAK clone 2A7	Upstate	mouse (mAb)	IP, IC	05-182
FAK	Upstate	rabbit	WB, IP, IH	06-543
FAK, BC3	Upstate	rabbit	IP, IC	06-446
FAK	BioSource	rabbit	IP, WB	AHO0502
FAK	BD PharMingen	rabbit	WB, IP	556368
FAK	BD Transduction	mouse (mAb)	WB, IP, IC, IH	610087
FAK	Cell Signaling	rabbit	WB, IH	3285
FAK	BioSource	rabbit	WB, IP, IC	AH0502
FAK	BioSource	rabbit	WB, IP, IC	AMO0672
FAK	Chemicon	rabbit	IP	AB1605
FAK	Chemicon	mouse (mAb)	WB, IP, IC	MAB2156
FAK (H-1)	Santa Cruz	mouse (mAb)	WB, IP, IC, IH	sc-1688
FAK (A-17)	Santa Cruz	rabbit	WB, IP, IC	sc-557
FAK (C-20)	Santa Cruz	rabbit	WB, IP, IC	sc-558
FAK (C-903)	Santa Cruz	rabbit	WB, IP, IC, IH	sc-932
Pyk2	Upstate	rabbit	WB, IP, IC	06-559
Pyk2, clone 74	Upstate	mouse (mAb)	WB, IP	05-488
Pyk2	Upstate	rabbit	WB, IP, IC	07-437
Pyk2	Cell Signaling	rabbit	WB, IP	3292
Pyk2	BD Transduction	mouse (mAb)	WB, IP, IC, IH	610548
Pyk2 (H-102)	Santa Cruz	mouse (mAb)	WB, IP, IC	sc-9019
Pyk2 (N-19)	Santa Cruz	rabbit	WB, IP, IC	sc-1514
Pyk2 (C-19)	Santa Cruz	rabbit	WB, IP, IC	sc-1515
c-Src, clone GD11	Upstate	mouse	WB, IP	05-184
c-Src, clone N6L	Upstate	rabbit (mAb)	WB	05-889
c-Src, clone NL19	Upstate	rabbit (mAb)	WB, IP	05-772
c-Src	BioSource	mouse (mAb)	WB	AHO1152
c-Src	BioSource	rabbit	WB	44-655G
c-Src	BioSource	rabbit	WB	44-656G
c-Src	Chemicon	sheep	WB, IP	CB769
c-Src, clone 36D10	Cell Signaling	rabbit (mAb)	WB, IP, IC, IH	2109
c-Src, clone L4A1	Cell Signaling	mouse (mAb)	WB, IP	2110
c-Src	Cell Signaling	rabbit	WB, IP, IC, IH	2108
c-Src (H-12)	Santa Cruz	mouse (mAb)	WB, IP, IC	sc-5266
c-Src (B-12)	Santa Cruz	mouse (mAb)	WB, IP, IC	sc-8056
c-Src (N-16)	Santa Cruz	rabbit	WB, IP, IC	sc-19
c-Src (Src 2)	Santa Cruz	rabbit	WB, IP, IC	sc-18

^aAbbreviation: mAb, monoclonal antibody; WB, western (immuno)blot; IP, immunoprecipitation; IC, immunocytochemistry; IH, immunohistochemistry.

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Table 14.7.2 Commercially Available Phospho-Specific Antibodies to FAK, Pyk2, and c-Src^a

Antibody	Company	Source	Application	Catalog number
FAK pY397	BioSource	rabbit	WB, IC, IH	44-624G
FAK pY397 clone 141-9	BioSource	rabbit (mAb)	WB, IC	44-625G
FAK pY407	BioSource	rabbit	WB, IC, IH	44-650G
FAK pY576	BioSource	rabbit	WB	44-652G
FAK pY577	BioSource	rabbit	WB, IC	44-614G
FAK pS722	BioSource	rabbit	WB	44-588
FAK pS732	BioSource	rabbit	WB	44-590G
FAK pS843	BioSource	rabbit	WB	44-594
FAK pY861	BioSource	rabbit	WB, IC	44-626G
FAK pS910	BioSource	rabbit	WB	44-596
FAK pY397 clone 14	BD/Transduction	mouse (mAb)	WB, IC	611722
FAK pY397 clone 18	BD/Transduction	mouse (mAb)	WB, IC	611806
FAK pY576/pY577	Cell Signaling	rabbit	WB	3281
FAK pY397	Santa Cruz	rabbit	WB, IC	sc-11765-R
FAK pY397	Santa Cruz	rabbit	WB, IC	sc-21868-R
FAK pY407	Santa Cruz	goat	WB, IC	sc-16664
FAK pY576	Santa Cruz	rabbit	WB, IC	sc-16563-R
FAK pY477	Santa Cruz	goat	WB, IC	sc-16665
FAK pY576/pY577	Santa Cruz	rabbit	WB, IC	sc-21831-R
FAK pY576/pY577	Santa Cruz	goat	WB, IC	sc-21831
FAK pS722	Santa Cruz	goat	WB, IC	sc-16662
FAK pY861	Santa Cruz	goat	WB, IC	sc-16663
FAK pS910	Santa Cruz	goat	WB, IC	sc-16666
FAK pY925	Santa Cruz	goat	WB, IC	sc-11766
FAK pY397	Chemicon	mouse (mAb)	WB, IC	MAB1144
FAK pY397	Upstate	rabbit	WB, IC	07-012
FAK pY576	Upstate	rabbit	WB, IC	07-157
Pyk2 pY402	BioSource	rabbit	WB, IH	44-618G
Pyk2 pY579	BioSource	rabbit	WB, IC	44-632G
Pyk2 pY579/pY580	BioSource	rabbit	WB	44-636G
Pyk2 pY580	BioSource	rabbit	WB	44-634G
Pyk2 pY881	BioSource	rabbit	WB, IH	44-620
Pyk2 pY402	Cell Signaling	rabbit	WB, IP	3291

continued

Table 14.7.2 Commercially Available Phospho-Specific Antibodies to FAK, Pyk2, and c-Src^a, *continued*

Antibody	Company	Source	Application	Catalog number
Pyk2 pY402	Santa Cruz	rabbit	WB, IC	sc-11767-R
Pyk2 pY579	Santa Cruz	goat	WB, IC	sc-16822
Pyk2 pY579/pY580	Santa Cruz	goat	WB, IC	sc-16824
Pyk2 pY580	Santa Cruz	goat	WB, IC	sc-16823
Pyk2 pY881	Santa Cruz	goat	WB, IC	sc-16825
Pyk2 pY402 clone RR102	Upstate	mouse (mAb)	IP	05-679
Active Src clone 28	BioSource	mouse (mAb)	WB, IC, IH	AHO0051
Src pY416	BioSource	rabbit	WB, IC	44-660G
Src pY527	BioSource	rabbit	WB, IH	44-662G
Src pY527 clone 31	BD/Transduction	mouse (mAb)	WB	612668
Src pY416	Cell Signaling	rabbit	WB, IC, IH	2101
Src pY416 clone 7G9	Cell Signaling	mouse (mAb)	WB, IP	2102
Non-phospho Src pY527	Cell Signaling	rabbit	WB	2107
Src pY527	Cell Signaling	rabbit	WB, IH	2105
Src pY527	Santa Cruz	goat	WB, IC	sc-16846
Src pY416 clone 2N8	Upstate	rabbit (mAb)	WB	05-857
Src pY416 clone 9A6	Upstate	mouse (mAb)	WB	05-677

^aAbbreviations: mAb, monoclonal antibody; WB, western (immuno)blot; IP Immunoprecipitation; IC, immunocytochemistry; IH, immunohistochemistry.

Reprobe membranes

12. For sequential probing of membranes with different antibodies, strip bound antibodies from membranes by incubating 15 min in western blot stripping buffer at 65°C. Wash membrane thoroughly with water.
13. Repeat immunoblotting procedure from step 4 to step 9.

Blots can be stripped two to three times to analyze FAK and Pyk2 immune complexes with various phospho-specific antibodies. It is recommended that a different species of primary antibody be used (rabbit, mouse, or goat) in the subsequent blotting analyses to ensure that signals detected are not from the first set of antibodies used to probe the membrane.

HAPTOTAXIS MOTILITY ASSAY: MATRIX-STIMULATED MIGRATION

FAK is rapidly activated via tyrosine phosphorylation and binds directly to c-Src, resulting in the formation of a FAK-Src signaling complex that leads to the activation of various downstream signaling cascades. In the preceding protocols, emphasis was placed on biochemical evaluations of FAK, Pyk2, or Src activation or phosphorylation after cell binding to extracellular matrix proteins. In addition to biochemical signaling changes

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PROTOCOL 4**

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inside cells, cell binding to matrix proteins such as fibronectin can result in enhanced cell spreading and the initiation of cell migration. Notably, FAK^{-/-} cells spread poorly and exhibit refractory motility responses in response to a fibronectin stimulus. Although it has been shown that FAK reexpression within FAK^{-/-} cells can reverse the motility defects, the molecular pathways through which FAK promotes cell motility and the inability of Pyk2 in FAK^{-/-} cells to function as a replacement for FAK remain active areas of research. There are various assays whereby cell motility can be measured. To specifically evaluate the contribution of integrin signaling, haptotaxis (a directed response of cells in a gradient of adhesion) motility assays are performed in the absence of serum where FAK signaling plays important roles in promoting efficient cell movement towards the extracellular matrix.

Materials

- FAK^{-/-} and FAK^{+/+} cells, subconfluent (see Support Protocol 1)
- Human plasma fibronectin (Sigma-Aldrich F2006): 2 to 10 µg/ml in replating and migration medium (see recipe), 37°C
- BSA-coating control: 10 µg BSA/ml in replating and migration medium (see recipe)
- Trypsin/EDTA: 0.25% (w/v) trypsin/1 mM EDTA (Invitrogen)
- Trypsin inhibitor solution (see recipe)
- PBS⁺⁺: phosphate-buffered saline (PBS; APPENDIX 2A) with 0.1 g/liter CaCl₂ and 0.5 mM MgCl₂
- Cell fixative: PBS with 1.85% (v/v) formaldehyde and 0.05% (v/v) glutaraldehyde
- 10% (w/v) crystal violet stain (Sigma) in ethanol
- 0.1 M sodium borate, pH 9.0
- Parafilm
- Millicell PCF chamber inserts, 8-µm pore (Millipore P1TP01250)
- Flat-tipped forceps
- 15-ml centrifuge tube
- Tabletop centrifuge
- Hemocytometer
- 24 well-tissue culture dishes (Costar)
- Cotton swabs
- Inverted light microscope
- Spectrophotometer with A₆₀₀ capability, optional
- Additional reagents and equipment for serum-starving cells (Support Protocol 2) and counting cells (UNIT 1.1)

Starve cells

1. Twenty-four hr before the motility assay, serum starve subconfluent FAK^{-/-} and FAK^{+/+} cells (see Support Protocol 2).

Cells should be subconfluent during starvation. High-density or contact-inhibited cells do not respond well to motility stimuli during the short time period of the assay.

Prepare assay chamber

2. Pipet 100 µl of human plasma fibronectin onto Parafilm. Carefully place Millicell PCF chamber inserts one at a time on top the fibronectin-containing droplets. Prepare three chambers per experimental point and include a BSA-coating control.

The use of flat tipped forceps is best for the handling of individual chambers.

Be sure to cover the entire membrane and to avoid bubbles as this will result in poor ligand coverage. This method is designed to coat just the under surface of the Millicell chamber with fibronectin.

3. Incubate chambers 2 hr at room temperature. Cover the Parafilm and chambers on the bench top with a plastic container to avoid evaporation.
4. Rinse each chamber gently in PBS and let dry for at least 10 min at room temperature.

Prepare cells

5. Wash serum-starved cells with 10 ml PBS and aspirate off. Add 2.5 ml trypsin/EDTA per dish. Tap side of plates to facilitate cell release.
6. Add 5 ml trypsin inhibitor solution warmed to 37°C. Transfer cells to 15-ml centrifuge tube. Centrifuge 3 min at $750 \times g$, 21°C, in a tabletop centrifuge and aspirate supernatant.
7. Resuspend cells to $\sim 2 \times 10^5$ cells/ml in 37°C replating and migration medium. Keep in suspension at least 30 min and rotate tube at least every 5 min to keep cells from clumping, settling, and sticking to the plastic.
8. During incubation period, count cells using a hemacytometer (*UNIT 1.1*) and dilute a suspension of cells to a density of 33×10^4 cells/ml in replating and migration medium

Perform migration assay

9. Add 400 μ l replating and migration medium to each experimental well of a 24-well tissue culture dish. Carefully place chambers into wells making sure there are no bubbles.
10. Add 300 μ l cell suspension to each chamber (total of 1×10^5 cells).
11. Incubate 3 to 4 hr at 37°C with 10% CO₂.

Fibroblasts will show maximal migration within this period.

Variations of this assay can be used to examine different types of motility. For example, chambers can be coated on the top and bottom to study random motility. Additionally, growth factors can be added to the lower chamber to investigate cell chemotaxis responses.

Fix, stain, and count cells

12. Fill two 24-well tissue culture plates with 0.5 ml PBS⁺⁺ and one with 0.5 ml cell fixative. Transfer chambers to PBS⁺⁺ and incubate 2 min to wash. Move chambers to fixative for 5 min then into fresh PBS⁺⁺ for 2 min.
13. Dilute 10% crystal violet stain stock 1:100 in 0.1 M sodium borate, pH 9.0. Place chambers into 0.5 ml of 0.1% crystal violet for 30 min.
14. Remove chambers from stain and wash repeatedly in a large volume of water (e.g., in a 500 ml beaker).
15. Use a cotton swab to wipe excess cells and stain from the top side of the chamber membrane while taking care not to touch the bottom side of the chamber membrane. Let chambers dry overnight.

Chambers are now ready for analysis. Cells that migrate to the bottom of the chamber towards the ligand will be stained purple with crystal violet while nonmigrating cells on the upper surface have been removed.

16. Count cells using a standard 10 \times objective on an inverted microscope. Use the lid of the 24-well plate to support the chamber. Count fields at 2, 4, 6, 8, 10, and 12 o'clock positions and in the center of the membrane.

If there are too many cells per field to be accurately counted, the crystal violet stain can be eluted from the membrane in 10% acetic acid and values can be obtained by measuring light absorption in a spectrophotometer at 600 nm (A_{600}).

Motility values are determined by the average number of cells per field when counting three chambers per experimental point. The BSA-coated chambers should contain less than 1% of the total as migratory cells.

ALTERNATE PROTOCOL 3

ANALYZING CELL MOTILITY USING PLASMID-TRANSFECTED CELLS

The haptotaxis protocol is a good way to evaluate integrin-stimulated signaling events affecting cell migration. In many instances, there is a need to test the role of particular signaling proteins in altering cell motility responses. This is best accomplished through the transient transfection of cells with plasmid expression vectors combined with the cotransfection of a marker such as β -galactosidase (*lac Z*) to identify the plasmid-transfected cells, the assumption being that the cells that have been transfected with the *lac Z* plasmid and show β -galactosidase activity have also taken up the cotransfected FAK plasmid. This protocol is used to show that FAK re-expression can rescue the motility defects of FAK^{-/-} cells.

Additional Materials (also see Basic Protocol 4)

- pcDNA3.1 FAK (contact Schlaepfer lab)
- FAK^{-/-} cells (see Support Protocol 1)
- pcDNA3.1 LacZ (Invitrogen)
- Phosphate-buffered saline (PBS; APPENDIX 2A)
- PBS⁺⁺: phosphate-buffered saline (APPENDIX 2A) with 0.1 g/liter CaCl₂ and 0.5 mM MgCl₂
- Opti-MEM I reduced-serum medium (Invitrogen)
- PLUS reagent (Invitrogen)
- Lipofectamine (Invitrogen)
- Cell growth medium (see recipe) containing 20% (v/v) FBS (instead of 10%)
- Starvation medium: Cell growth medium (see recipe) with FBS reduced to 0.5%
- Lac Z staining solution (see recipe)
- Additional reagents and equipment for bacterial transformation (Seidman et al., 1997), plasmid miniprep (Engbrecht et al., 1991), and DNA quantification (APPENDIX 3D)

Prepare plasmids

1. Prepare high purity supercoiled plasmid DNA from bacteria (see Seidman et al., 1997; Engbrecht et al., 1991; and APPENDIX 3D). Combine 5 μ g of pcDNA3.1 FAK with 3 μ g pcDNA3.1 LacZ for each 10-cm plate.

Make sure the DNA is sterile by using alcohol precipitation.

Maximum amount of DNA per 10-cm plate transfection is 8.0 μ g.

Prepare cells

2. Plate 7.5×10^5 FAK^{-/-} cells onto gelatin-coated 10-cm plates (see Support Protocol 1) and let grow overnight at 37°C.
3. Aspirate medium and wash once with PBS to remove serum.
4. Add 4 ml of Opti-MEM I reduced-serum medium and place cells in the 37°C incubator for 30 to 45 min.

Prepare transfection mix

5. Aliquot 0.5 ml Opti-MEM I reduced-serum medium into two 1.5-ml microcentrifuge tubes (label tubes A and B).
6. Add 8 μ g plasmid DNA (FAK plus LacZ) to tube A and resuspend by tapping with your fingers.

7. Add 48 μ l PLUS reagent to the DNA in tube A and mix by tapping.
8. Add 32 μ l Lipofectamine reagent to tube B. Mix by tapping and incubate for 15 min.
9. Add contents of tube B to tube A and mix by tapping. Incubate for 30 min at 21°C.

IMPORTANT NOTE: *Do not mix by pipetting at this step.*

Transfect cells

10. Add the DNA/lipid complexes from step 9 to the cells from step 4 and allow cell transfection to proceed for 5 hr at 37°C in the 10% CO₂ incubator.
11. Add 5 ml cell growth medium containing 20% FBS to stop transfection. Continue incubation to 24 hr without changing the medium.

Prepare cells for motility assays

12. To set up cells for motility assays, wash with 10 ml PBS and place in 10 ml serum starvation medium and incubate overnight at 37°C in 10% CO₂.
13. Use cells for motility experiments 48 hr after transfection to ensure good protein expression from plasmids.
14. Proceed with the motility assay (Basic Protocol 4, steps 2 to 11).
15. Save excess cells not used in the motility assay to make protein lysates (Support Protocol 3) and to analyze by SDS-PAGE (UNIT 6.1) followed by immunoblotting (Basic Protocol 3 and UNIT 6.2).

This is required in order to verify transient protein overexpression (i.e., FAK and Lac Z) in the population of cells used for the motility assay.

Fix and count cells

16. After haptotaxis assays have proceeded for 3 to 4 hr, fill two 24-well tissue culture plates with 0.5 ml PBS⁺⁺ and one with 0.5 ml cell fixative. Transfer chambers to PBS⁺⁺ and incubate for 2 min (wash). Move chambers to fixative for 5 min then into fresh PBS⁺⁺ for 2 min. Repeat final PBS wash.

It is important to not leave cells in fixative for too long as this will result in less β -galactosidase activity.

17. Use a cotton swab to wipe excess cells from the top side of the chamber membrane while taking care not to touch the bottom side of the chamber membrane. Do not let chambers get too dry.
18. Add 0.5 ml Lac Z staining solution to wells of a 24-well tissue culture plate and incubate chambers 2 to 12 hr at 37°C or until blue-green color is observed.
19. Rinse chambers by gently immersing them in PBS and enumerate the blue β -gal positive cells under light microscopy.
20. To store plates fix chambers 10 min with 1 ml 10% formalin in PBS at room temperature, rinse with PBS, and store in PBS up to 1 week at 4°C.

It is assumed that the cells that have been transfected with the Lac Z plasmid and show β -galactosidase activity have also taken up the cotransfected FAK plasmid. If the protein of interest is tagged with a fluorescent marker such as green fluorescent protein, then the direct identification of transfected cells is facilitated, making it possible to observe the behavior of such cells in time-lapse imaging studies (see Basic Protocol 5).

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SCRATCH-WOUND HEALING ASSAY WITH TIME-LAPSE IMAGING

Wound healing assays (*UNIT 12.4*) are ideal for visualizing cellular dynamics during stimulated migration. In particular, time-lapse imaging allows for analysis of lamellipodia extension and membrane ruffling into the wounded area. Wound healing assays do not, however, address directional motility because the cells are plated at high density and movement is limited into the wound. Random migration assays can be performed to address directional motility by plating cells at low density and following the imaging protocol outlined below.

Materials

Extracellular matrix molecule (ECM) of interest (e.g., 2 μ g fibronectin/ml PBS)
70% confluent 24-hr serum-starved cells (see Support Protocol 2)
DMEM with and without 0.5 μ g/ml mitomycin-C
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Serum, optional
Mitomycin-C (Sigma)
Medium 199 (Invitrogen) with 0.5 μ g/ml mitomycin-C
Mineral oil (Sigma)
25-mm glass coverslips (1 oz., Fisher) and 6-well tissue culture plates *or*
35-mm Biotech delta-T dishes (Fisher)
Forceps
1- to 10- μ l micropipet tips
Transfer pipets, sterile
Inverted microscope with 20 \times objective, 37°C heated stage, and
acquisition/analysis software (e.g., Improvion Openlab;
<https://www.improvision.com>)
Etched-grid coverslips (Bellco), optional

Coat coverslips

1. Place 25-mm round glass coverslips into wells of a 6-well tissue culture plate. Pre-coat glass coverslip or delta-T dishes with matrix molecule of interest (e.g., 2 μ g fibronectin/ml PBS) for 2 hr at 37°C.

Other ECM molecules may be prepared in the same concentrations in PBS as fibronectin.

Too high a ligand concentration will inhibit cell motility. If migration is slow or non-existent, try decreasing the ligand concentration.

Add cells

2. Prepare a cell suspension of 1×10^6 70% confluent 24 hr serum-starved cells/ml in DMEM (see Basic Protocol 1, steps 4 to 5).

Serum starve cells for 24 hr before initiating wound healing assay.

Cells should be ~70% confluent during starvation.

3. Wash coverslips or delta-T dishes with PBS and plate 2×10^6 cells onto coverslips in a 6-well tissue culture plate or directly onto the delta-T dish.

Coverslips in 6-well tissue culture plates are used if subsequent procedures include an imaging chamber. Biotech delta-T dishes are self-contained.

4. Incubate at 37°C with 10% CO₂ for 2 hr.

The incubation time can be adjusted to accommodate other cells types if needed. This step is to facilitate equal cell adhesion and spreading on the matrix-coated glass surface.

Wound the cultures

5. Remove plates with coverslips from incubator. Using forceps to stabilize the coverslip in the well, carefully scratch the confluent monolayer of cells down the center using a micropipet tip.

If using a delta-T dish, use a similar technique to scratch the center of the glass insert while holding the dish steady.

This step is critical and will require practice in order to achieve consistent wounds. In order to reliably compare results from experiment to experiment, the wound distance must be equivalent each time.

6. Rinse the coverslip/delta-T dish three times with 2 ml PBS to remove loose cells.

If the cell monolayer is not washed effectively after scratching, loose cells can settle into the wounded area and result in an inaccurate interpretation of wound healing ability.

7. Place coverslip in fresh DMEM medium with 0.5 $\mu\text{g/ml}$ mitomycin-C and incubate at 37°C with 10% CO₂ for 1 hr.

Add 0.5 $\mu\text{g/ml}$ mitomycin-C to all medium changes from this point. This will inhibit cell mitogenesis and, therefore, control for wound closure due to cell proliferation.

Serum can be added (1% to 5% v/v, depending on cell type and required stimulation) at this point to stimulate cell motility if needed.

Assess motility

8. Aspirate medium and move coverslip into imaging chamber (if using the delta-T dishes, the imaging chamber is the unit itself). Add 1 ml of medium 199 with 0.5 $\mu\text{g/ml}$ mitomycin-C (with serum addition as needed).
9. Carefully layer 0.5 ml mineral oil on top of the medium to prevent evaporation during imaging and place imaging chamber/delta-T dish in heated stage on the microscope and set to 37°C.
10. Using a 10 \times or 20 \times objective, center the wound such that the image captured will show both sides of the cell monolayer flanking the wounded region. Using Improvise Openlab or comparable acquisition software, begin the time-lapse sequence and collect one image every 2 to 5 min for 12 to 15 hr or until the wound is closed.

Alternatively, if time-lapse software is not available, etched-grid coverslips can be used (Bellco). These coverslips have a grid system that allows tracking and measuring wound closure over time with a light microscope while leaving the coverslips in the incubator in between phase-image collection.

IMMUNOLocalIZATION OF FOCAL ADHESION PROTEINS

Immunostaining is the best approach to determining whether a protein of interest is localized to integrin-enriched focal adhesions. For valid interpretation of the staining results, two controls are essential: (1) immunoblot evaluation of the antibody against the protein before immunostaining and (2) cells costained with markers for focal adhesion proteins. The antibody to be used is suitable for cell staining analyses if it yields one band by immunoblotting analyses of whole cell lysates. The presence of multiple bands, even though the main one is the strongest, easily leads to misinterpretation of results. The most commonly used focal adhesion markers are vinculin and paxillin. Some of the best markers for activated focal adhesions are antibodies that recognize tyrosine-397-phosphorylated FAK or active Src-family members.

BASIC PROTOCOL 6

**Signal
Transduction:
Protein
Phosphorylation**

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Materials

- Matrix-coating substrates: prepared according to manufacturer's directions and diluted (commonly to 10 $\mu\text{g/ml}$) in PBS (*APPENDIX 2A*)
- Fibronectin, human plasma (Roche)
 - Laminin, human placenta (Sigma)
 - Vitronectin, human plasma (Sigma)
 - Poly-L-lysine (70,000–150,000 or 150,000–300,000; Sigma)
 - Collagen, Type I, human placenta (Calbiochem)
 - Collagen, Type II, bovine (Calbiochem)
 - Collagen, Type IV, human placenta (Calbiochem)
 - Collagen, Type V, human (Calbiochem)
- FAK^{-/-} and FAK^{+/+} cells (see Support Protocol 1)
- Replating and migration medium (see recipe), warm *or* growth medium (see recipe), 37°C
- Phosphate-buffered saline (PBS; *APPENDIX 2A*)
- 3.8% (w/v) paraformaldehyde fixative (see recipe)
- Acetone, cold (stored at -20°C) *or*
- 0.5% (v/v) Triton X-100/0.05% (v/v) Tween 20/PBS *or*
- 0.2% Triton X-100/PBS *or*
- 0.2% (v/v) Triton X-100/3.8% (w/v) paraformaldehyde/PBS *or*
- 0.1% (v/v) Triton X-100/0.1% (w/v) sodium citrate *or*
- Methanol, cold
- Blocking antibody (e.g., ChromPure donkey IgG, unconjugated; Jackson ImmunoResearch) *or*
- 2% (w/v) BSA in PBS
- 1 to 10 $\mu\text{g/ml}$ PBS (*APPENDIX 2A*) primary antibodies for focal adhesion markers
- anti-paxillin (ZO35; Zymed/Invitrogen)
 - anti-vinculin (VIN-11-5; Sigma)
 - anti-FAK (clone #77; BD-Transduction)
 - anti-FAK (Ab-1; LabVision)
 - anti-phospho Y397FAK (BioSource)
 - anti-active Src family members (clone #28; BioSource)
- Secondary antibodies: e.g., fluorescein (FITC)-conjugated donkey antibodies (excitation/emission maxima 492/520 nm; Jackson ImmunoResearch) *or*
- FITC-conjugate
- anti-mouse IgG
 - anti-mouse IgM
 - anti-rabbit IgG
 - anti-goat IgG
 - anti-rat IgG
- Rhodamine X (RRX)-conjugated donkey antibodies (excitation/emission maxima 570/590 nm; Jackson ImmunoResearch) *or* Rhodamine X (RRX)-conjugated
- anti-mouse IgG
 - anti-mouse IgM
 - anti-rabbit IgG
 - anti-goat IgG
 - anti-rat IgG
- Hoechst 33342 (excitation/emission maxima 350/460; Molecular Probes)
- Vectashield mounting medium (Vector)
- Nail polish, clear
- 12-mm round coverslips, German glass (Bellco Glass)
- 4-well tissue culture plates (Nunc)
- 12-well tissue culture plates (Costar)
- 18- to 22-G needle with a slightly bent tip
- Flat-ended forceps with beveled, unserrated tips, stainless steel (Millipore)

6-well tissue culture plates
Rotating platform shaker
Vacuum source
Porcelain spot plates with 12 cavities (CoorsTek)
Light microscope with fluorescence excitation and detecting capability

1. Coat 12-mm round coverslips with matrix-coating substrate (e.g., by incubating with 10 µg/ml fibronectin 45 min at room temperature).

The most commonly used adhesion molecule to induce formation of focal contacts is fibronectin, but the same volumes and concentrations are recommended for laminin, vitronectin, poly-L-lysine, and collagen. The coating time should be extended to 2 hr for collagen, and the coverslips should be washed with PBS to neutralize the acidity of the collagen solutions.

Plate cells

2. Prepare FAK^{-/-} and FAK^{+/+} cell suspension of 2×10^4 cells/ml in 37°C replating and migration medium or growth medium (see Basic Protocol 1, steps 5 to 6), and add 0.5 ml cell suspension per well of a 4-well tissue culture plate containing a coated coverslip.

Blocking with BSA is not required because cells will not attach to the uncoated glass surface.

At this concentration the adherent cells will be spread apart.

If the purpose is to determine activation or localization of the protein by adhesion molecule of interest, replating and migration medium should be used, otherwise growth medium is appropriate.

The well size of 4-well tissue culture plates is the same as in 24-well plates. However, the wells are not so deep, and it is easier to take coverslips out.

3. Incubate at 37°C for desired time.

Activation (phosphorylation) of the focal adhesion proteins is the highest during cell spreading. For fibroblasts, the best time to capture localization of the activated proteins in focal adhesions is ~1 hr after plating. At that time most of cells will be spread. Some cells will still be spreading and often both types can be visualized within a single field.

Activated focal adhesion proteins at the leading edge of migrating cell are best visualized 5 to 6 hr after wounding a cell monolayer with a micropipet tip. Activation is higher in the presence of serum. The most commonly used serum concentration is 10%, but one could also see activation at much lower serum concentrations.

Fix and permeabilize cells

4. Aspirate medium and rinse briefly 2 to 3 times in ~1 ml PBS by adding ~1 ml PBS and aspirating or decanting it.

For most cells

- 5a. Add ~1 ml 3.8% paraformaldehyde/PBS, pH 7.4 to fix cells. Incubate 20 min at room temperature and rinse with PBS as above.
- 6a. Transfer coverslips into a porcelain spot plate filled with cold acetone (stored at -20°C) to permeabilize the cells. Keep on ice for 10 min. Remove acetone and add ~1 ml PBS to rehydrate for 3 to 5 min.

Transfer of coverslips to a porcelain plate is necessary only when using acetone, which will dissolve plastic tissue culture dishes.

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Fixation and permeabilization are the most critical steps for successful immunostaining. Even for the same protein, this step can differ from cell type to cell type and from antibody to antibody. Most focal adhesion proteins are detectable when cells are fixed in paraformaldehyde and permeabilized in acetone. If the staining is weak or negative, changing fixation and permeabilization should be the first step to troubleshoot.

For weakly staining cells (alternative 1)

- 5b. Add ~1 ml 3.8% (w/v) paraformaldehyde/PBS, pH 7.4 to fix cells. Incubate 20 min at room temperature.
- 6b. Remove paraformaldehyde and add ~1 ml 0.5% (v/v) Triton X-100 + 0.05% (v/v) Tween 20/PBS for 2 min to permeabilize the cells.

For weakly staining cells (alternative 2)

- 5c. Add ~1 ml 3.8% (w/v) paraformaldehyde/PBS, pH 7.4 to fix cells. Incubate 20 min at room temperature.
- 6c. Remove paraformaldehyde and add ~1 ml 0.2% (v/v) Triton X-100/PBS for 2 min to permeabilize the cells.

For weakly staining cells (alternative 3)

- 5d. Add ~1 ml 0.2% (v/v) Triton X-100/3.8% (w/v) paraformaldehyde/PBS and incubate 2 min at room temperature to fix and permeabilize cells. Rinse several times with 3.8% paraformaldehyde/PBS as in step 4.
- 6d. Add ~1 ml 3.8% paraformaldehyde/PBS and continue fixing for 20 min at room temperature.

For weakly staining cells (alternative 4)

- 5e. Add ~1 ml 3.8% (w/v) paraformaldehyde/PBS, pH 7.4 to fix cells. Incubate 20 min at room temperature.
- 6e. Add ~1 ml 0.1% (v/v) Triton X-100/0.1% (w/v) sodium citrate and keep 2 to 5 min on ice to permeabilize the cells.

For weakly staining cells (alternative 5)

- 5f. Transfer coverslips into a porcelain spot plate filled with cold acetone (stored at -20°C) to fix and permeabilize cells. Keep on ice for 10 min.

In some cases in the more common protocol described in step 5a, paraformaldehyde fixation can interfere with staining. In these cases coverslips are directly exposed to acetone, which can also act as a fixative.

- 6f. Remove acetone and add ~1 ml PBS to rehydrate for 3 to 5 min.

Staining of actin stress fibers is impossible when fixation/permeabilization is done with ONLY acetone or methanol (alternatives 5 and 6).

For weakly staining cells (alternative 6)

- 5g. Add ice-cold methanol (stored at -20°C) to fix and permeabilize cells. Keep on ice for 10 min.
- 6g. Aspirate methanol, rinse briefly in ~1 ml PBS and leave in PBS for 3 to 5 min to rehydrate.

Staining of actin stress fibers is impossible when fixation/permeabilization is done with ONLY acetone or methanol (alternatives 5 and 6).

Block and immunostain cells

7. Place a 30- μ l drop of PBS containing blocking antibodies and primary antibodies for focal adhesion markers on the inner side of 12-well plate lid (facing up), and place the coverslip with fixed and permeabilized cells on top, with the attached cells facing the drop of antibody solution. Cover with the bottom of the plate and incubate 1 hr at room temperature or as long as overnight at 4°C, depending on convenience and the effectiveness of staining with various samples and procedures.

Using a 30- μ l drop of PBS containing blocking antibodies and primary antibodies for focal adhesion markers on the inner side of a 12-well plate lid helps prevent evaporation, ensure equal exposure, and use less antibody solution.

Flip coverslip over the 30- μ l drop of PBS containing blocking and primary antibodies using an 18- to 22-G needle with a slightly bent tip to lift and flat-ended forceps to hold and transfer the coverslip. Sharp-ended forceps tend to crack the coverslip. Alternatively, if the cell type is sensitive to flipping the coverslips for incubations and cells detach, utilize a humidified chamber (to avoid evaporation) and add 50 μ l of antibody directly on top of the coverslips.

Blocking is done with whole IgG of the host animal for the secondary antibody. For example, the primary antibody is made in mouse. If the secondary antibody is fluorescein-conjugated donkey anti-mouse, donkey whole IgG should be used for blocking. If the secondary antibody is fluorescein-conjugated goat anti-mouse, goat whole IgG should be used for blocking, etc. Blocking antibodies are sold in concentrations \sim 10 mg/ml. Recommended dilution for blocking purposes is 1:100 (\sim 100 μ g/ml). To keep costs low, the authors use secondary antibodies that are all made in donkey, requiring only one reagent (ChromPure donkey whole IgG) for blocking purposes.

Alternatively, blocking can be performed with 2% (w/v) BSA in PBS. In this case, blocking is done at the same time as sample incubation with the primary antibody, saving time. BSA blocking is less specific than IgG, and in rare cases BSA might be too strong a blocking agent.

Dilution of primary antibodies can vary and should be optimized for each antibody. Generally a range of 1 to 10 μ g/ml is effective. A starting point for optimization would be a 1:100 dilution of a commercial antibody, which is usually sold in concentrations 0.1 to 1 mg/ml.

For co-immunostaining, incubate samples with both primary antibodies and with blocking antibodies at the same time. If incubations are for several hours, samples can be left at room temperature. If incubation is performed overnight, the incubation should be at 4°C.

8. Lift the coverslip by injecting 150 μ l PBS under it, and place it in a well of a 6-well tissue culture plate, attached cells facing up. Wash in three changes of \sim 3 ml PBS 5 min each time at \sim 50 rpm on rotating platform shaker.

Smaller size wells (e.g., wells of 12- or 24-well plates) do not allow sufficient movement of PBS to wash unbound antibodies from the samples.

9. Place a 30- μ l drop of PBS containing secondary antibodies and 10 μ g/ml Hoechst 33342 on the inner side of a 12-well plate lid (facing up), and place the coverslip with fixed and permeabilized cells on top with the attached cells facing the drop of antibody solution. Cover with plate bottom and incubate 40 min at room temperature.

Hoechst 33342 binds DNA, and it is an excellent bright nuclear marker visible at UV excitation wavelengths. It does not leak to green or red channels. It is of immense help for orientation and focusing of samples during microscopy.

For visualization of actin stress fibers, fluorescent phalloidin conjugate is added together with secondary antibody for costaining of two antigens: one is detected by the primary antibody, and the other (actin stress fibers) is detected directly by fluorophore-conjugated phalloidin.

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10. Wash in three changes of ~3 ml PBS three times 5 min each time at ~50 rpm on a rotating platform shaker.
11. Mount on microscope slides in Vectashield mounting medium. Aspirate excess mounting medium under the coverslip using a Pasteur pipet attached to vacuum. To prevent sliding, fix the edge of the coverslip with clear nail polish. Wait 15 to 20 min for the nail polish to dry, then analyze samples under the microscope.

Samples can be preserved at 4°C in the dark for varying lengths of time, depending on the strength of the staining. For example, actin staining can be preserved for a month or longer. Weak staining will fade beyond recognition within several days. The authors recommend analysis within 2 to 3 days after staining.

**ALTERNATE
PROTOCOL 4**

IMMUNOLocalIZATION OF ACTIN STRESS FIBERS

Visualization of actin stress fibers with fluorescent phalloidin, a toxin with high binding affinity for actin filaments, is easy and almost certainly yields dazzling results. Unlike indirect immunostaining with antibodies, phalloidin staining is strong, and the contrast between stained and unstained areas is high. Because focal adhesions are located at the end of actin stress fibers, phalloidin staining is also used as a localization marker and as a quick assay to assess cell shape. Costaining actin stress fibers with fluorescent phalloidin-conjugate and DNA-binding Hoechst dye can be a rewarding experience for a new investigator who is performing immunofluorescence cell staining for the first time.

Additional Materials (also see Basic Protocol 6)

0.4 to 1 U/ml (10 to 30 nM) fluorescein-conjugated phalloidin (Molecular Probes)
or rhodamine-conjugated phalloidin (Molecular Probes) in PBS

Rotating platform shaker

Vacuum source

Prepare cells

1. Coat coverslips, prepare FAK^{-/-} and FAK^{+/+} cell suspension, and plate the cells (Basic Protocol 6, steps 1 to 3).
2. Incubate at 37°C for desired time.

Fix and permeabilize cells

3. Aspirate medium and rinse briefly 2 to 3 times in ~1 ml PBS by adding ~1 ml PBS and aspirating or decanting it.
4. Add ~1 ml 3.8% paraformaldehyde/PBS, pH 7.4, to fix cells. Incubate 20 min at room temperature and rinse with PBS as above.
5. Transfer coverslips into a porcelain spot plate filled with cold acetone (stored at -20°C) to permeabilize the cells. Keep on ice for 10 min. Remove acetone and add ~1 ml PBS to rehydrate for 3 to 5 min.
6. Place a 30- μ l drop containing 0.4 to 1 U/ml (10 to 30 nM) fluorescein-conjugated phalloidin with 10 μ g/ml Hoechst 33342 in PBS on the inner side of a 12-well tissue culture plate lid (facing up), and place the coverslip with fixed and permeabilized cells on top with the attached cells facing the drop of solution. Cover with the plate bottom and incubate 30 min at room temperature.

If dissolved as recommended by the manufacturer (Molecular Probes), 0.4 to 1 U/ml (10 to 30 nM) phalloidin is 1:200 to 1:500.

Rhodamine-conjugated phalloidin is an alternative to fluorescein-conjugated phalloidin.

If costaining with some other protein, phalloidin should be added together with the secondary antibody (see Basic Protocol 6).

7. Wash in three changes of ~3 ml PBS 5 min each time at ~50 rpm on a rotating platform shaker.
8. Mount on microscope slides in Vectashield mounting medium. Aspirate excess mounting medium under the coverslip using a Pasteur pipet attached to vacuum. To prevent sliding, fix the edge of the coverslip with nail polish. Wait 15 to 20 min for the nail polish to dry, then analyze samples under the microscope.

Samples can be preserved at 4°C in the dark for varying lengths of time depending on the strength of the staining. For example, actin staining can be preserved for a month or longer. Weak staining will fade beyond recognition within several days. The authors recommend analysis within 2 to 3 days after staining.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

BSA blocking buffer

20 mM Tris-Cl, pH 7.4
150 mM NaCl
2% (w/v) BSA (Fraction V)
0.05% (v/v) Tween 20
0.05% (w/v) sodium azide
Store up to 3 months at 4°C

Cell growth medium

DMEM high-glucose with L-glutamine (Invitrogen)
10% (v/v) FBS
1 mM sodium pyruvate (Invitrogen)
0.1 mM MEM nonessential amino acids (Invitrogen)
10 U/ml penicillin and 10 µg/ml streptomycin (Invitrogen; 100× stock)
Sterilize by passing through a 0.22-µm filter
Store up to 2 weeks at 4°C

Coomassie blue stain

25% (v/v) isopropanol (2-propanol)
10% (v/v) glacial acetic acid
0.03% (w/v) Coomassie G-250 (BioRad)
Store up to 1 year at 21°C

Destaining solution

10% (v/v) isopropanol (2-propanol)
10% (v/v) glacial acetic acid
Store up to 1 year at 21°C

FAK-Pyk2 kinase buffer

20 mM HEPES, pH 7.4
10% (v/v) glycerol
10 mM MgCl₂
10 mM MnCl₂
150 mM NaCl
Store up to 6 months at 4°C

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HNTG buffer

50 mM HEPES, pH 7.4
0.1% (v/v) Triton X-100
150 mM NaCl
10% (v/v) glycerol
Store up to 6 months at 4°C

Lac Z staining solution

70 ml H₂O
10 ml of 10× PBS (1× final)
10 ml of 50 mM potassium ferricyanide (5 mM final)
10 ml of 50 mM potassium ferrocyanide (5 mM final)
0.2 ml of 1 M MgCl₂ (2 mM final)
Store up to 2 weeks at 4°C
Just before use, add 1 ml of 20 mg/ml Xgal per 20 ml of staining solution (1 mg/ml final). Make a stock solution of 20 mg/ml X-gal in dimethylformamide (DMF).
Store at -20°C in the dark.

NOTE: Use a glass pipet to measure DMF solutions as it will dissolve plastics.

Laemmli SDS sample buffer (2×)

125 mM Tris·Cl, pH 6.8
20% (v/v) glycerol
4% (w/v) SDS
0.006% (w/v) bromophenol blue (Fisher Biotech)
3.5% (v/v) 2-mercaptoethanol
Store up to 1 week at 4°C or 6 months at -20°C

Magnesium/ATP cocktail

75 mM MgCl₂
500 μM ATP
20 mM MOPS, pH 7.2
25 mM β-glycerol phosphate
5 mM EGTA
1 mM sodium orthovanadate
1 mM dithiothreitol
Store up to 1 week at 4°C

Paraformaldehyde fixative, 3.8% (w/v)

Add 3.8 g paraformaldehyde (Sigma) to 100 ml PBS (APPENDIX 2A), heat, and stir to dissolve. When temperature reaches 50°C, add ~100 μl 1 N NaOH, and continue stirring until temperature reaches 60°C. Remove from the heating plate, continue stirring until it reaches room temperature and filter to remove fine precipitate. Store up to 10 days at 21°C.

Once made, paraformaldehyde fixative is ready to use for up to 10 days. The exact percentage of paraformaldehyde is not crucial; it is sufficient to be somewhere between 3.8% and 4.0%. Similarly, the exact pH does not matter, if it is somewhere in a physiological range (7.2 to 7.6). Paraformaldehyde from different manufacturers or of different purity might require different amount of NaOH to adjust the pH.

Replating and migration medium

500 ml DMEM
0.5% (w/v) BSA (add 20 ml 12.5% BSA solution)
1 mM sodium pyruvate (Invitrogen)

continued

0.1 mM MEM nonessential amino acids (Invitrogen)
Sterilize by passing through a 0.22- μ m filter
Store up to 1 month at 4°C

RIPA cell lysis buffer

1% (v/v) Triton X-100
1% (v/v) sodium deoxycholate
0.1% (w/v) SDS
50 mM HEPES, pH 7.4
150 mM NaCl
10% (v/v) glycerol
1.5 mM MgCl₂
1 mM EGTA
10 mM sodium pyrophosphate
100 mM sodium fluoride
1 mM sodium orthovanadate (add dropwise)
10 μ g/ml aprotinin
10 μ g/ml leupeptin
Sterilize by passing through a 0.22- μ m filter
Store up to 1 week at 4°C

Add ingredients slowly to water. Start over if buffer becomes brown after sodium orthovanadate addition.

Alternatively, a protease inhibitor cocktail (e.g. Sigma P2714) may be used instead of aprotinin and leupeptin.

Src kinase buffer

20 mM PIPES, pH 7.0
10 mM MnCl₂
1 mM DTT
Store up to 6 months at 4°C

Tris-buffered saline with Tween (TBST)

50 mM Tris·Cl, pH 7.5
150 mM NaCl
0.05% (v/v) Tween 20
Store up to 6 months at 4°C

Triton lysis buffer

RIPA cell lysis buffer (see recipe) without sodium deoxycholate and SDS addition.
Store up to 1 week at 4°C

Trypsin inhibitor solution

500 ml DMEM
125 mg soybean trypsin inhibitor (Worthington)
Dissolve by stirring
0.25% (w/v) BSA (add 10 ml of 12.5% w/v BSA solution)
Sterilize by passing through a 0.22 μ m filter
Prepare fresh

Western blot stripping buffer

100 mM Tris·Cl, pH 6.8
2% (w/v) SDS
100 mM 2-mercaptoethanol
Store up to 3 months at 21°C

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COMMENTARY

Background Information

FAK was first identified in 1992 as a highly tyrosine-phosphorylated protein associated with cellular focal adhesions, i.e., the integrin-associated cell attachment points with the extracellular matrix (Parsons, 2003). At that time, it was shown that changes in cell adhesion or integrin clustering could result in the increased phosphorylation of tyrosine in FAK (Guan and Shalloway, 1992; Hanks et al., 1992; Kornberg et al., 1992; Schaller et al., 1992). Today we know that this activation event is much more complicated than the simple binding of FAK to integrins leading to intermolecular FAK phosphorylation after clustering (Schlaepfer and Mitra, 2004). For instance, evidence to date does not strongly support direct binding of FAK to integrins. Instead, FAK is associated with integrin cytoplasmic tails through binding to integrin-associated proteins such as paxillin and talin. Additionally, integrins can activate Src-family protein-tyrosine kinases in a manner that is independent of FAK. It is known that cell binding to fibronectin promotes FAK and c-Src activation and the formation of a FAK-Src signaling complex, and these facts support the conclusion that the events are related. Current research is focused on deciphering the molecular mechanisms through which FAK and c-Src are activated by integrins, and the methods outlined in this unit comprise the foundation for these studies.

Additional complexity in this integrin signaling linkage comes from the studies of the FAK-related kinase, Pyk2, which is highly expressed in FAK^{-/-} cells (Sieg et al., 1998). Stimulation of FAK^{-/-} cells by replating on fibronectin promotes the activation of Src-family tyrosine kinases and the enhanced tyrosine phosphorylation of Pyk2. However, neither Pyk2 nor c-Src strongly colocalize with FAK^{-/-} cell focal adhesions, and these cells do not correctly spread and migrate like wild-type FAK^{+/+} fibroblasts (Sieg et al., 1999; Klingbeil et al., 2001). The localization of FAK to particular integrin signaling sites is important for fibronectin-stimulated cell motility, the molecular mechanisms of which are still under investigation.

What the authors have tried to accomplish in this unit is to consolidate related protocols on how to measure changes in FAK, c-Src, and Pyk2 activity after fibronectin-mediated integrin stimulation through the use of *in vitro* kinase assays, phospho-specific blotting, or im-

munolocalization techniques. These assays involve using FAK^{-/-} and FAK^{+/+} cells. The handling or preparation of these cells prior to performing biochemical activation assays is the same as the procedures used in evaluating the properties of these cells in cell migration assays. In this manner, cause and effect relationships can be established between integrin-stimulated signaling events and the modulation of a complex biological process such as cell motility. Alternate protocols that have been successfully used by other investigators include the use of antibodies or recombinant ligands for particular integrin subunits to facilitate binding or clustering and intracellular signaling (Giancotti and Ruoslahti, 1999). As cells usually contain multiple integrin α/β pairs that can bind fibronectin, and current research has shown that sequence-specific differences between integrin cytoplasmic domains facilitates the binding or activation of distinct intracellular signaling proteins (Liu et al., 1999; de Virgilio et al., 2004), antibody/ligand activation of integrins is a powerful means to study integrin signaling. However, antibody-mediated clustering of integrins is usually a weaker stimulus compared to cell binding and spreading on matrix proteins. Therefore, if a recombinant ligand can be purified and immobilized to facilitate specific integrin engagement and cell binding, this can be effectively used to study the signaling linkage between particular integrins, the actin cytoskeleton, and cell motility.

Critical Parameters and Troubleshooting

Maintenance of FAK^{+/+} AND FAK^{-/-} fibroblasts and replating assays for signaling studies

It is critical to use well maintained FAK^{+/+} and FAK^{-/-} cells for all experiments in this unit. Because the fibroblasts may exhibit altered morphology and premature senescence when overly confluent or at high passage number, cells need to be subcultured consistently and used within the first 15 passages from the cells available from American Type Culture Collection (ATCC). To prevent poor attachment to the extracellular matrices when cells are replated, avoid overtrypsinization and always make fresh trypsin inhibitor solution. Further, serum starvation is an important step in allowing maximal cell response and activation of focal adhesion molecules to stimuli (e.g., fibronectin). This

is also critical for successful motility assays (Basic Protocol 4) and scratch-wound healing assays (Basic Protocol 5).

In vitro kinase assay

It is important that a control antibody is included with immunoprecipitation experiments to accurately interpret *in vitro* kinase assay results. For example, preimmune serum can serve as the control for a rabbit antiserum. Further, the species (e.g., rabbit, mouse, goat, rat, or sheep) of antibody used in immunoprecipitation should be carefully considered if the immunoprecipitates will be analyzed by subsequent immunoblotting. HRP-conjugated secondary antibodies will recognize the immunoprecipitating antibody as well as the primary immunoblotting antibody of the same species, resulting in very high background around the 60-kDa protein region. As this is region where c-Src is present on gels, non-specific antibody cross-reactivity can complicate co-immunoprecipitation experiments and c-Src immunodetection.

Immunoblotting with FAK/Pyk2 phospho-specific antibodies

For the detection of FAK and Pyk2 phosphorylation with phospho-specific antibodies, the most critical issue is the specificity and quality of the antibody. Ideally, the phospho-specific antibody should recognize only the phosphorylated form of the target protein on a specific site. However, in the experience of the authors, some phospho-specific antibodies for FAK can cross-react with corresponding sites on Pyk2 and may even react with other phosphorylation sites on the target protein. It is for this reason that results should be double-checked using complementary means such as phospho-specific antibodies from various manufacturers and alternate procedures (e.g., *in vitro* kinase assays). When reprobing with different phospho-specific antibodies, it is critical to ensure that stripping or removal of previous antibodies is complete. This can be done by performing the ECL reaction and exposing film after every blot stripping to confirm that no residual signal remains.

Haptotaxis motility assays

As confluent cells do not respond to extracellular matrix stimuli, it is essential to maintain a low cell confluency when performing the haptotaxis assay. Additionally, chamber coating is key to a successful and interpretable motility assay. Uneven or improper chamber coating will result in experimental variability within single chambers as well as through-

out the experiment. Potential causes of uneven coating include denatured or sticky extracellular proteins and the presence of bubbles underneath the chambers during coating.

Scratch-wound healing assays

After generating the wound, it is important to ensure that no residual cells settle in the scratch area. This would severely complicate interpretation of the assay and can be avoided by thorough washing after creating the scratch wound. Additionally, wound-closure measurements must be compared to the original wound distance for each individual coverslip due to experimental scratch variations.

Immunostaining

The most important issues for visualization of focal adhesion molecules with immunofluorescent staining are (1) fixation and permeabilization of cells and (2) specificity of the staining antibody. Optimized fixation allows the preservation and localization of the antigen as well as cell morphology, while proper permeabilization allows access to the antigen. To achieve specificity for the immunostaining, the primary antibody should recognize only its target antigen, and proper controls (e.g., preimmune serum or secondary antibody only) should be included to optimize the staining conditions and to reduce background. See Table 14.7.3 for troubleshooting the experiments described in this unit.

The authors do not recommend use of preimmune serum as a control for staining specificity. The more appropriate control is a Western blot of whole cell lysate made from the same cells treated in the same manner. If multiple bands are visible, the antibody is not reliable for immunostaining. A control for secondary antibody is not necessary if whole IgG of the same species in which the secondary antibody was generated is used as a blocking agent together with the primary antibody.

Anticipated Results

In attached and serum-starved FAK^{+/+} and FAK^{-/-} cells, FAK and Pyk2, respectively, display high basal levels of tyrosine phosphorylation and moderate level of kinase activity. In suspended cells, phosphorylation of tyrosine in both FAK and Pyk2 is greatly reduced. Replating cells on fibronectin, but not poly-L-lysine, increases tyrosine phosphorylation on both FAK and Pyk2 in FAK^{+/+} and FAK^{-/-} cells. Immunoblotting with phospho-specific antibodies reveals that FAK and Pyk2 can be phosphorylated on

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Table 14.7.3 Troubleshooting Guide for Common Problems Encountered in These Assays

Problem	Possible cause	Solution
<i>Cell replating</i>		
Cells slow to adhere and spread on matrix	Too much trypsin used to prepare cell suspension	Use limited trypsin/EDTA treatment at 37°C and tap plates to remove cells Make fresh trypsin inhibitor solution
<i>In vitro kinase assay</i>		
No or low levels of substrate labeling	Old [γ - ³² P]ATP	Order fresh [γ - ³² P]ATP
	Kinase denatured or degraded	Add fresh protease inhibitor to lysis buffer. Use freshly prepared lysates for immunoprecipitation Avoid repeated freeze-thaw of samples Use polyclonal antibodies to target protein for immunoprecipitation
High background	Carryover of cell debris following preclearance	Avoid disturbing cell debris when transferring supernatant during lysate preparation
	Incomplete washing	Completely resuspend beads at each wash
<i>Phospho-specific immunoblotting</i>		
Phospho-specific antibody recognizing nonphosphorylated form of protein in immunoblotting	Too much antigen or antibody present	Optimize the amount of lysates and concentration of antibody used. Reduce antibody incubation time and increase washing
<i>Haptotaxis motility assay</i>		
Migratory cells too dense to quantify	Assay went too long	Decrease assay time
	Too many cells were plated	Plate fewer cells
Inconsistent cell migration	Improper chamber coating	Avoid bubbles underneath chamber
	Matrix protein precipitated out of solution	Confirm the pH of medium used to resuspend matrix protein
<i>Scratch-wound healing</i>		
Cells do not close the wound	Matrix protein concentration is too high	Decrease matrix protein concentration
	Cells are not plated as a confluent monolayer	Increase cell density
	Cells require serum stimulation	Add 1% to 5% serum during assay
Cells close the wound too quickly	Too many cells	Decrease cell density
	Elevated cell proliferation	Add mitomycin C to block cell division

continued

Table 14.7.3 Troubleshooting Guide for Common Problems Encountered in These Assays, *continued*

Problem	Possible cause	Solution
<i>Immunolocalization</i>		
No staining	Primary antibody is not binding to epitope	Use different primary antibody Try another fixation procedure Try a different secondary antibody
Staining looks non-specific or high background	Complications due to fixation procedure	Try another fixation procedure
	Primary antibody does not cross-react across species	Change primary antibody
	Antigen target is expressed at low levels	Increase primary antibody incubation from 1 hr to overnight. Increase concentration of primary antibody Use biotin-avidin enhancement
Identical pattern observed for multiple proteins	Primary antibodies are from same species	Change one primary antibody to a different species
Signal "leaking" through microscope filters	Too high concentration of primary or secondary antibody	Decrease antibody concentrations or increase number of washes

multiple tyrosine residues following replating onto fibronectin matrix, and, following integrin activation, Src-associated *in vitro* kinase assays demonstrate that FAK and Pyk2 can transiently form signaling complexes with Src.

In haptotaxis and scratch-wound healing assays, FAK^{-/-} cells display impaired migration compared to FAK^{+/+} cells. The defective cell motility of FAK^{-/-} cells cannot be compensated by the presence of high levels of Pyk2, but it can be rescued by transiently re-expressing FAK. Furthermore, FAK can be detected via immunofluorescence staining that prominently and specifically localize to focal adhesions. In fact, some of the best markers of activated focal adhesions are antibodies that recognize FAK phosphorylated on tyrosine 397. Finally, strong activation of FAK can be detected in focal adhesions about 1 hr after replating fibroblasts onto extracellular matrix as well as at the leading edge of migration 5 to 6 hr following a scratch wound.

Time Considerations

Cells need to be serum starved (Support Protocol 2) for 16 to 24 hr before replating (Basic Protocol 1), haptotaxis motility (Basic

Protocol 4), and scratch-wound healing (Basic Protocol 5) assays. Cells are kept in suspension for at least 45 min or replated for 1 to 3 hr before whole-cell lysates are collected, which takes 1 hr to complete (Support Protocol 3).

FAK, Pyk2, and c-Src can be captured from whole cell lysates by immunoprecipitation in 4 to 5 hr (Support Protocol 4), and can be further analyzed through *in vitro* kinase assay (Basic Protocol 2) or processed by immunoblotting for their phosphorylation and activation status (Basic Protocol 3). In these protocols SDS-PAGE gel electrophoresis takes 1 to 1.5 hr, gel transfer onto membrane takes 1.5 to 2 hr, and immunoblotting is best when membranes are incubated in primary antibodies overnight. Therefore, these biochemical assays will take a total of 3 days.

Following serum starvation (16 to 24 hr), the haptotaxis assay (Basic Protocol 4) takes ~8 hr to complete. It takes 2.5 to 3 hr to prepare the chambers and 1 to 1.5 hr to prepare cells (depending on how many different experimental groups are being analyzed), but these two items can be done concurrently. Cells migrate for 3 hr followed by an additional 1 to 2 hr

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to fix and stain chambers. Microscopic quantification of migratory cells can take several hours.

To transfect FAK^{+/+} and FAK^{-/-} cells (Alternate Protocol 3), cells need to be plated the previous day; preparation of transfection mix requires 45 min to 1 hr, and the transfection takes 5 hr. Cells are grown for an additional 48 hr to allow maximal protein expression and need to be serum starved for 24 hr before haptotaxis assay.

For the scratch-wound healing assay (Basic Protocol 5), cells are serum starved for 16 to 24 hr, plated on a cover slip for 2 hr, scratch wounded, and incubated for 1 hr after wounding. Live images are collected for 12 to 15 hr. The scratch-wound healing assay spans 32 to 45 hrs, but requires only 2 to 3 hr of hands-on time. For immunolocalization of focal adhesion proteins (Basic Protocol 6), immunofluorescence staining can be performed in ~7 hr.

Internet Resources

<http://www.cellmigration.org/index.shtml>

This cell migration gateway represents a unique collaboration between the Cell Migration Consortium (CMC) and Nature Publishing Group (NPG) and is designed to facilitate navigation through the complex world of cell migration research.

<http://www.signaling-gateway.org>

This signaling gateway represents a unique collaboration between academia and scientific publishing and is designed to facilitate navigation of the complex world of research into cellular signaling. Information and data presented here are freely available to all.

Literature Cited

- de Virgilio, M., Kiosses, W.B., and Shattil, S.J. 2004. Proximal, selective, and dynamic interactions between integrin $\alpha_{11b}\beta_3$ and protein tyrosine kinases in living cells. *J. Cell Biol.* 165:305-311.
- Engbrecht, J., Brent, R., and Kaderbhai, M.A. 1991. Minipreps of plasmid DNA. In *Current Protocols in Molecular Biology* (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 1.6.1-1.6.10. John Wiley & Sons, Hoboken, N.J.
- Giancotti, F.G. and Ruoslahti, E. 1999. Integrin signaling. *Science* 285:1028-1032.
- Guan, J.L. and Shalloway, D. 1992. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature* 358:690-692.
- Hanks, S.K., Calalb, M.B., Harper, M.C., and Patel, S.K. 1992. Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. *Proc. Natl. Acad. Sci. U.S.A.* 89:8487-8491.
- Hynes, R.O. 2002. Integrins: Bidirectional, allosteric signaling machines. *Cell* 110:673-687.
- Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Natkatsuji, N., Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T., and Aizawa, S. 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from *fak*-deficient mice. *Nature* 377:539-544.
- Klingbeil, C.K., Hauck, C.R., Hsia, D.A., Jones, K.C., Reider, S.R., and Schlaepfer, D.D. 2001. Targeting Pyk2 to β_1 -integrin-containing focal contacts rescues fibronectin-stimulated signaling and haptotactic motility defects of focal adhesion kinase-null cells. *J. Cell Biol.* 152:97-110.
- Kornberg, L., Earp, H.S., Parsons, J.T., Schaller, M., and Juliano, R.I. 1992. Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *J. Biol. Chem.* 267:23439-23442.
- Liu, S., Thomas, S.M., Woodside, D.G., Rose, D.M., Kiosses, W.B., Pfaff, M., and Ginsberg, M.H. 1999. Binding of paxillin to α_4 integrins modifies integrin-dependent biological responses. *Nature* 402:676-681.
- Parsons, J.T. 2003. Focal adhesion kinase: The first ten years. *J. Cell Sci.* 116:1409-1416.
- Schaller, M.D., Borgman, C.A., Cobb, B.A., Vines, R.R., Reynolds, A.B., and Parsons, J.T. 1992. pp125^{fak} a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. U.S.A.* 89:5192-5196.
- Schlaepfer, D.D. and Mitra, S.K., 2004. Multiple connections link FAK to cell motility and invasion. *Curr. Opin. Genet. Dev.* 14:92-101.
- Schlaepfer, D.D., Hauck, C.R., and Sieg, D.J. 1999. Signaling through focal adhesion kinase. *Prog. Biophys. Mol. Biol.* 71:435-478.
- Schwartz, M.A. 2001. Integrin signaling revisited. *Trends Cell Biol.* 11:466-70.
- Seidman, C.E., Struhl, K., Sheen, J., and Jessen, T. 1997. Introduction of plasmid DNA into cells. In *Current Protocols in Molecular Biology* (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.) pp. 1.8.1-1.8.10. John Wiley & Sons, Hoboken, N.J.
- Sieg, D.J., Ilic, D., Jones, K.C., Damsky, C.H., Hunter, T., and Schlaepfer, D.D. 1998. Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin-stimulated signaling events but Pyk2 does not fully function to enhance FAK^{-/-} cell migration. *EMBO J.* 17:5933-5947.
- Sieg, D.J., Hauck, C.R., and Schlaepfer, D.D. 1999. Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J. Cell Sci.* 112:2677-2691.

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