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Absolute Quantification of Phosphorylation on the Kinase Activation Loop of Cellular Focal Adhesion Kinase by Stable Isotope Dilution Liquid Chromatography/Mass Spectrometry

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Abstract

A vital point of convergence for many signaling pathways at cellular focal adhesions is the interaction of two non-receptor tyrosine kinases, focal adhesion kinase (FAK) and Src. The binding of Src to FAK leads to the phosphorylation of Y⁵⁷⁶ and Y⁵⁷⁷, located within the activation loop domain of FAK. However, it has not been possible previously to determine the absolute quantitative relationship between phosphorylated and non-phosphorylated forms of this activation loop domain in cells undergoing normal metabolism. We have developed a stable isotope dilution liquid chromatography-multiple reaction monitoring/mass spectrometry (LC-MRM/MS) technique that allows such determinations to be made. Isotopically labeled and phosphorylated FAK protein standards were synthesized and used to control for loss during immunoprecipitation of FAK. A control tryptic peptide, representing an unmodified region of FAK, was employed to monitor the mass balance of post-translational modifications (PTMs) on the activation loop domain. Absolute quantification was conducted using stable isotope labeled peptide standards with four endogenous amino acid overhangs at the trypsin digestion sites of both the amino and carboxy terminus. The LC-MRM/MS method was rigorously validated using in vitro kinase assays and employed to conduct absolute quantification of FAK phosphorylation in normal mouse embryo fibroblasts (MEFs). This methodology will have particular utility for biomarker studies of kinase-inhibiting anti-cancer drugs and for quantitative proteomic investigations that examine kinase- and phosphatase-mediated cellular signal transduction pathways.

INTRODUCTION

Cellular Src (c-Src) is a non-receptor tyrosine kinase that signals downstream of multiple cell surface receptors including receptor tyrosine kinases, G-protein coupled receptors, and integrins, linking its activity to growth factors, androgens, cell adhesion, and cytokines.^{1,2} The ability of c-Src to impinge on the cellular cytoskeleton is largely dependant upon its interaction with focal adhesion kinase (FAK), another non-receptor tyrosine kinase that acts as a signaling scaffold in the regulation of adhesion dynamics and cell motility.³⁻⁶ The critical importance of FAK is evident from studies showing that FAK null MEFs display reduced spreading and motility, which are defects that can be rescued by re-expression of wild-type FAK but not by

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signaling deficient mutants⁷⁻⁹ Additionally, reduced FAK phosphorylation following Src inhibition correlates with decreased cell proliferation and motility. This means that FAK phosphorylation can be used as a biomarker to monitor the efficacy Src inhibitors as anti-cancer drugs.¹⁰⁻¹²

FAK is a 125-kDa protein containing an N-terminal FERM (protein 4.1, band F ezrin, radixin, and moesin homology) domain, a central kinase domain, proline-rich regions (PRR), and a C-terminal focal-adhesion targeting domain (FAT domain). The biological activity of FAK is governed to a large degree by tyrosine phosphorylation. Autophosphorylation of FAK at Y³⁹⁷ creates a binding site for the SH2 domain of Src.¹³ This interaction leads to the activation of both FAK and Src and coincides with the phosphorylation by Src of additional FAK tyrosine residues including Y⁴⁰⁷, Y⁵⁷⁶, Y⁵⁷⁷, Y⁸⁶¹, and Y⁹²⁵ (Figure 1).¹³⁻¹⁶ Adjacent FAK Y⁵⁷⁶/Y⁵⁷⁷ residues lie within the activation loop of the kinase domain, and phosphorylation of these residues results in enhanced FAK activity.

Stable isotope dilution LC-MRM/MS-based methodology provides the most specific and sensitive technique to conduct absolute quantification of peptides.^{17;18} This methodology can be implemented after isolation of cellular FAK, followed by trypsin digestion to yield the relevant tryptic peptide containing Y⁵⁷⁶/Y⁵⁷⁷ (Figure 1).¹⁹ It was necessary to first immunoprecipitate FAK from whole cell lysates in order to reduce background signal and allow interference-free quantitation of FAK Y⁵⁷⁶/Y⁵⁷⁷ phosphorylation. Protein losses during isolation procedures such as immunoprecipitation is a concern for quantitative LC-MS methodology that is only beginning to be addressed.^{20;21} However, little attention has been given to developing rigorous methodology for quantifying site-specific cellular protein phosphorylation following immunoprecipitation. In this investigation, we describe the use of heavy isotope labeled phosphorylated recombinant FAK standards that were added to the cell lysate and immunoprecipitated along with endogenous FAK, to determine the recovery from immunoprecipitation. Quantification of a tryptic peptide (E⁹⁵⁶VGLALR⁹⁶²) representing a segment of FAK that could not be phosphorylated was used as an additional control (Figure 1). This made it possible to compare the amount of phosphorylation on the activation loop region with the amount of control peptide. In conjunction with the more traditional application of heavy isotope-labeled peptide standards,^{22;23} it was then possible to correct for losses during immunoprecipitation and so determine the absolute amount of FAK together with its Y⁵⁷⁶- and Y⁵⁷⁷-phosphorylated forms.

EXPERIMENTAL SECTION

Reagents and Materials

The gene for human FAK (PTK2- α) in the Gateway® entry vector pENTER221 was from Invitrogen (Carlsbad, CA). The pDEST26 and pEXP1 plasmids, nickel-chelating resin (Probond™), the cell free expression system, and recombinant full-length c-Src and FAK were also from Invitrogen. Heavy isotope-labeled amino acids were from Cambridge Isotope Laboratories (Andover, MA). Dialysis cassettes were from Pierce Biotechnology (Rockford, IL). Anti-FAK beads (clone 4.47, agarose conjugate beads) were from Millipore (Billerica, MA). Protease inhibitors were from Roche Applied Sciences (Indianapolis, IN). The autosampler was a CTC pal from Leap technologies (Carrboro, NC), while the LC pump was an ExpressLC100 system from Eksigent Technologies (Dublin, CA). LC grade water and acetonitrile (ACN) were from Burdick and Jackson (Muskegon, MI), while Suprapur® formic acid was from EMD Chemical (Gibbstown, NJ). The reversed phase column (50 × 1 mm internal diameter) was custom made using extended C18 (300 Å, 3.5 μm) by Agilent Technologies (Santa Clara, CA). An LTQ mass spectrometer (Thermo Fisher, San Jose, CA) was employed for quantitative analyses. It is an extremely fast scanning linear trap instrument capable of quantifying peptides by LC-MRM/MS with high sensitivity, precision, and

accuracy.¹⁸ Excalibur (Version 2.2) software was from ThermoFisher Scientific (San Jose, CA). All standard peptides were synthesized and quantified using amino acid analysis by AnaSpec, Inc. (San Jose, CA).

Cell-free synthesis of stable isotope labeled FAK protein ([¹³C¹⁵N]-FAK)

A full length FAK construct (from cDNA clone MGC: 34721) correlating to the human gene, protein tyrosine kinase 2 alpha (PTK2- α), was obtained from commercial sources. Creation of an expression clone suitable for cell free production was accomplished following manufacturer's instructions using restriction free recombination technology (Gateway®) and plasmids pDEST26 then pEXP1. The resultant expression clone contained FAK N-terminally tagged with a 6XHIS epitope downstream of the T7 promoter and ribosomal binding site. FAK protein labeled with [¹³C₆¹⁵N₄]-arginine, [¹³C₉]-tyrosine, [¹³C₆¹⁵N₂]-lysine, and [¹³C₆¹⁵N]-leucine was produced using the expressway cell free expression system, supplied with heavy isotope-labeled amino acids, similar to published techniques.^{21;24} The cell free reaction was conducted according to the manufacturer's directions and the subsequent [¹³C¹⁵N]-labeled 6XHIS-FAK ([¹³C¹⁵N]-FAK) was purified on a 2 mL nickel-chelating resin under non-denaturing conditions. Following a wash step, [¹³C¹⁵N]-FAK was eluted and stored in the presence of 10 % glycerol at -80 °C until further use. A portion of this reaction mixture was resolved on an SDS-PAGE gel and stained with Coomassie blue. The prominent band corresponding to ~ 125 kDa (as compared to a protein mass marker) was picked for LC-MS analysis. In gel trypsin digestion was performed and the isotopic purity of [¹³C¹⁵N]-FAK was determined by LC-tandem MS (MS/MS).

In vitro tyrosine phosphorylation of [¹³C¹⁵N]-FAK

In order to phosphorylate [¹³C¹⁵N]-FAK using purified recombinant Src in an *in vitro* kinase reaction, it was first necessary to denature [¹³C¹⁵N]-FAK and allow refolding during buffer exchange. To accomplish this, [¹³C¹⁵N]-FAK was mixed 1:1 with a denaturing buffer consisting of 6 M urea, 2 M thiourea, and 10 mM dithiothreitol (DTT), and heated at 37 °C for 30 min. Following heating, the solution containing denatured [¹³C¹⁵N]-FAK was dialyzed for 6 h at 4 °C against oxidizing dialysis buffer { 1 mM oxidized glutathione, 40 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 270 mM sucrose, 100 μ M EGTA, 100 μ M Na₃VO₄, and 0.03 % Brij-35 } using a 10,000 MWCO filter dialysis cassette. After 6 h, the dialysis buffer was replaced and dialysis was allowed to continue for an additional 6 h. The refolded and buffer exchanged [¹³C¹⁵N]-FAK was then split into portions, and each portion was adjusted to contain, 1 mM ATP, 1 X kinase buffer, and recombinant Src (33 nM of full length recombinant c-Src). Following incubation at 37 °C for 30 min, all portions were pooled and stored at -80 °C. Both full scanning LC-electrospray ionization (ESI)/MS/MS and LC-MRM/MS were used to ascertain the extent of Y⁵⁷⁶/Y⁵⁷⁷ phosphorylation on [¹³C¹⁵N]-FAK.

Cell culture

Y⁵²⁹FSrc, vector-only (normal) MEFs²⁵ or FAK^{-/-} MEFs ("R3" clonal isolate) were used.⁷ Cells were cultured in Dulbecco's Modified Eagle's Medium with 10 % fetal bovine serum, and penicillin/streptomycin. Cultures were kept below 90 % confluence on 100 mm culture dishes until harvesting, at which point they were allowed to grow to full confluence.

Immunoprecipitation

Immediately, or following cell treatment, media was removed from a confluent monolayer of adherent cells. To a plate, 500 μ L of 70 °C LDS-reducing buffer (2 X Invitrogen stock, 10 mM DTT, 2 X complete protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN) and 2 mM activated Na₃VO₄ (phosphatase inhibitor) was applied. The cells were then quickly harvested using a cell scraper. [¹³C¹⁵N]-FAK { 12 μ L stock, corresponding to 0.514 pmol of

unphosphorylated- $^{13}\text{C}^{15}\text{N}$ -FAK, 0.404 pmol of mono-phosphorylated-(pY⁵⁷⁶)[$^{13}\text{C}^{15}\text{N}$]-FAK, and 0.118 pmol of bis-phosphorylated-(pY⁵⁷⁶/pY⁵⁷⁷)-[$^{13}\text{C}^{15}\text{N}$]-FAK} was added to the lysing cells and the plate was re-scraped. Following scraping, the cell lysate was immediately brought to 1 mL with 70 °C cytoskeletal extraction buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM activated Na₃VO₄, 1 % Triton X-100, 0.1 % sodium dodecyl sulfate, 0.5 % sodium deoxycholate, and 1 X protease inhibitor cocktail) and heated for 10 min at 70 °C. After heating, the lysate was further diluted to 4 mL with cytoskeletal extraction buffer. The diluted cell lysate was then homogenized by passing all 4 mL of the cell lysate through a 21 gauge needle 10 times. Cell lysates were further cleared of debris by centrifugation at 3000 × g for 10 min. A 100 µL portion was removed and used to quantify the amount of total protein using the Bradford method.²⁶ To the remaining supernatant, 15 µL anti-FAK beads were added and all samples were gently nutated at 4 °C for 12 h. Immunoprecipitation beads were then washed with 2 mL of cold cytoskeletal extraction buffer and nutation was conducted at 4 °C for 15 min. Beads were spun down at 1500 × g, supernatant removed, and washed two additional times as before. Beads were finally washed in 2.0 mL of cold phosphate-buffered saline (PBS) three times in order to reduce detergent and protease inhibitor concentrations in the samples. Following the last PBS wash, 50 µL of elution buffer (0.3 % formic acid in 50:50 H₂O:ACN) was added to beads and samples were heated to 50 °C for 5 min in order to elute FAK. The solution was then carefully removed to a new 500 µL vial. This step was repeated once and the two eluents were pooled. The resulting solution of eluted FAK in 100 µL ACN/H₂O (1:1, v/v) with 0.3 % formic acid was dried to ~ 1 µL using vacuum centrifugation. Samples were resuspended in 40 µL of digestion buffer (25 mM ammonium bicarbonate, 10 mM EDTA, and 10 mM DTT) and 10 µL of [$^{13}\text{C}^{15}\text{N}$]-peptide standard mixture (100 fmol/µL stock mixture of FAK activation loop peptides from unphosphorylated-FAK (GLSR YMEDSTY⁵⁷⁶Y⁵⁷⁷[$^{13}\text{C}_6^{15}\text{N}_2$]-KASKG), pY⁵⁷⁶-FAK (GLSR YMEDSTpY⁵⁷⁶Y⁵⁷⁷[$^{13}\text{C}_6^{15}\text{N}_2$]-KASKG), pY⁵⁷⁶/pY⁵⁷⁷-FAK (GLSR YMEDSTpY⁵⁷⁶pY⁵⁷⁷[$^{13}\text{C}_6^{15}\text{N}_2$]-KASKG), and the control segment peptide (PMVKE [$^{13}\text{C}_5^{15}\text{N}$]VGLALRTLTA). Tryptic digestion was then performed in solution following the procedure described previously.¹⁹ The experimental workflow for quantification following immunoprecipitation is shown in Figure 2.

LC-MS conditions

A full sample loop injection was made and peptides were loaded onto a micro-analytical C18 column at 25 µL/min using 100 % buffer A [ACN/H₂O (0.1:20, v/v) with 0.1 % formic acid]. The mobile phase was diverted to waste for the first 5 min at these conditions in order to remove salts, following which, the flow rate was reduced to 14 µL/min and peptides were eluted over a 30 min gradient from 0 % to 40 % B {ACN/H₂O (19:1, v/v) with 0.1 % formic acid}. After gradient elution, the column was washed at 25 µL/min using 100 % buffer B for 10 min, and then equilibrated with 100 % buffer A for 10 min. A blank injection was made between all biological samples to check for any carry over from the previous injection. The LTQ mass spectrometer was operated in the positive ion mode using ESI with a capillary temperature of 200 °C. Nitrogen was used as the sheath gas at a setting of 41 (arbitrary units) and as an auxiliary gas set at 12 (arbitrary units). All MRM transitions were monitored with a parent ion mass window of 2.5 Da and a product ion mass window of 2.5 Da. Helium was used as the collision gas with the collision induced dissociation (CID) energy set at 20 % and activation energy (Act. Q) of 0.35 (arbitrary units). LC-MRM/MS analysis was conducted within a single segment spanning the entire gradient elution. MS/MS experiments used a full scan mass window of m/z 500 to 1500 m/z with data dependant acquisition CID conducted on the top 5 parent ions.

Analysis of FAK activation loop phosphorylation status using LC-MRM/MS

Peptides spanning the tryptic region containing FAK activation loop Y⁵⁷⁶ and Y⁵⁷⁷ (Y⁵⁷⁰MEDSTYYK⁵⁷⁸) with the endogenous four amino acid overhangs at both the amino and carboxy terminus were synthesized as standards for unphosphorylated FAK (GLSRY⁵⁷⁰MEDSTY⁵⁷⁶Y⁵⁷⁷K⁵⁷⁸ASKG), pY⁵⁷⁶-FAK (GLSRY⁵⁷⁰MEDSTpY⁵⁷⁶Y⁵⁷⁷K⁵⁷⁸ASKG), and pY⁵⁷⁶pY⁵⁷⁷-FAK (GLSRY⁵⁷⁰MEDSTpY⁵⁷⁶pY⁵⁷⁷K⁵⁷⁸ASKG). The corresponding [¹³C¹⁵N]-labeled forms were also synthesized in which K⁵⁷⁸ was replaced with [¹³C₆¹⁵N₂]K⁵⁷⁸ (Figure 1). In addition, a control segment of FAK, representing an unmodified tryptic peptide, was synthesized as unlabeled (PMVKE⁹⁵⁶VGLALR⁹⁶²TLLA) and labeled (PMVKE⁹⁵⁶[¹³C₅¹⁵N]VGLALR⁹⁶²TLLA) forms, which also each had four amino acids overhangs at both the amino and carboxy terminus (Figure 1). All peptides were initially dissolved in 10 mM EDTA/H₂O. Following trypsin digestion, LC-MRM/MS was employed to monitor a total of twelve peptides resulting from the hydrolysis of endogenous FAK, [¹³C¹⁵N]-FAK, and [¹³C¹⁵N]-labeled peptide standards in a single LC-MRM/MS analysis (Table 1). MRM transitions for the unphosphorylated Y⁵⁷⁶/Y⁵⁷⁷ FAK tryptic peptide spanning residues Y⁵⁷⁰ to K⁵⁷⁸ monitored the MH₂²⁺ parent to the y₇ product ion: YMEDSTYYK (*m/z* 600.25 → 905.40), [¹³C₉]YMEDST[¹³C₉]Y[¹³C₉]Y[¹³C₆¹⁵N₂]K (*m/z* 617.25 → 930.40), and YMEDSTYY[¹³C₆¹⁵N₂]K (*m/z* 604.25 → 913.40). Mono-phosphorylated (pY⁵⁷⁶) transitions monitored the MH₂²⁺ parent to the y₇ and y₇ (-H₂O) product ions: YMEDSTpYYK (*m/z* 640.25 → 985.35 and 967.35), [¹³C₉]YMEDST[¹³C₉]pY[¹³C₉]Y[¹³C₆¹⁵N₂]K (*m/z* 657.25 → 1010.36 and 992.36), and YMEDSTpYY[¹³C₆¹⁵N₂]K (*m/z* 644.25 → 993.36 and 975.36). Bis-phosphorylated (pY⁵⁷⁶/pY⁵⁷⁷) transitions monitored the MH₂²⁺ parent to the y₇ product ion: YMEDSTpYpYK (*m/z* 680.25 → 1047.31), [¹³C₉]YMEDST[¹³C₉]pY[¹³C₉]pY[¹³C₆¹⁵N₂]K (*m/z* 697.25 → 1072.32), and YMEDSTpYpY[¹³C₆¹⁵N₂]K (*m/z* 684.25 → 1055.32). Lastly, the control segment peptide spanning FAK residues E⁹⁵⁶ to R⁹⁶² monitored the MH₂²⁺ parent to the y₃ product ion: EVGLALR (*m/z* 379.23 → 359.24), EVG[¹³C₆¹⁵N]LA[¹³C₆¹⁵N]L [¹³C₆¹⁵N₄]R (*m/z* 382.23 → 359.24), and E[¹³C₅¹⁵N]VGLALR (*m/z* 391.23 → 376.24).

Preparation of standard curves, data processing, and normalization

Standard curves were prepared by mixing known amounts of unlabeled peptide standards, a known amount of [¹³C¹⁵N]-FAK, and fixed amounts of the [¹³C¹⁵N]-peptide standards. For immunoprecipitation experiments, the [¹³C¹⁵N]-FAK standard was used to determine percent recovery over a range of 2 % to 50 %. LC-MRM/MS ion chromatograms were quantified using linear regression analysis methods supplied in Excalibur software with no weighting of calibration points. The response ratios of endogenous and [¹³C¹⁵N]-FAK to their heavy isotope-labeled standards were calculated by interpolation using their respective standard curves to determine the pmol amount of endogenous FAK and percent immunoprecipitation recovery for [¹³C¹⁵N]-FAK. Two corrections for immunoprecipitation efficiency, an unphosphorylated percent recovery and a phosphorylated percent recovery were calculated. These correction values were applied to the raw amounts of endogenous FAK unphosphorylated peptide (using the calculated unphosphorylated % recovery), and mono-(pY⁵⁷⁶) and bis-phosphorylated (pY⁵⁷⁶/pY⁵⁷⁷) FAK peptides (using the phosphorylated % recovery). Hence, cellular FAK concentrations were corrected for recovery through the immunoprecipitation step (Supplemental Figures 1 and 2).

Validation Experiments

In a total reaction volume of 200 μL, recombinant FAK (258.8 nM) and c-Src (12.8 nM) were incubated in the presence of ATP (1 mM) at 37 °C. At various time points during a 120 min reaction, 5 μL of the reaction was removed and spiked into 35 μL 70 °C digestion buffer (25 mM ammonium bicarbonate, 10 mM EDTA, and 10 mM DTT) and kept at 70 °C for an

additional 10 min. Following the collection of the last time point, all samples received 10 μL of [$^{13}\text{C}^{15}\text{N}$]-peptide standard mixture (100 fmol/ μL stock). Tryptic digestion and LC-MRM/MS analyses were then conducted as described above. In order to validate the immunoprecipitation procedure and immunoprecipitation correction, a reaction was again carried out *in vitro* as described above, but, the entire reaction was stopped at 5 min by heating to 70 $^{\circ}\text{C}$ for 10 min. A 10 μL portion of this reaction together with 12 μL of [$^{13}\text{C}^{15}\text{N}$]-FAK solution were added to a lysing dish of FAK-/- MEFs, or a 10 μL portion of this reaction, together with 6 μL of the [$^{13}\text{C}^{15}\text{N}$]-FAK solution were added to two lysing dishes, and pooled. Immunoprecipitation was conducted as above. In parallel, 12 μL of heat-inactivated [$^{13}\text{C}^{15}\text{N}$]-FAK solution was mixed with a 10 μL portion of the *in vitro* kinase reaction in a vial. Following the elution of FAK from the immune-complex, all samples were processed side-by-side, as described above. LC-MRM/MS analyses were then conducted on all the samples.

RESULTS AND DISCUSSION

In vitro synthesis and phosphorylation of [$^{13}\text{C}^{15}\text{N}$]-FAK

Following the *in vitro* synthesis of FAK, a portion of the reaction mixture was resolved on an SDS-PAGE gel and Coomassie stained. Staining revealed a prominent band at ~ 125 kDa (data not shown). Trypsin digestion of this band followed by LC-MS/MS analysis verified that *in vitro* synthesis of FAK resulted in expression of a protein that was isotopically labeled with [$^{13}\text{C}_6^{15}\text{N}_4$]arginine, [$^{13}\text{C}_9$]tyrosine, [$^{13}\text{C}_6^{15}\text{N}_2$]lysine, and [$^{13}\text{C}_6^{15}\text{N}$]leucine. The tryptic peptide containing the FAK activation loop domain sites of phosphorylation (Y⁵⁷⁶ and Y⁵⁷⁷) Y⁵⁷⁰MEDSTYYK⁵⁷⁸ was [$^{13}\text{C}_{33}^{15}\text{N}_2$]-labeled. The control tryptic peptide, E⁹⁵⁶VGLALR⁹⁶², was [$^{13}\text{C}_{18}^{15}\text{N}_6$]-labeled. Full scanning MS/MS and MRM/MS showed that the unlabeled species were present at <0.1 % and partially labeled species were not detected. In order to phosphorylate this *in vitro* synthesized FAK, it was first necessary to denature the protein and allow refolding to occur in an oxidizing environment. Following the de-nature/re-nature step, [$^{13}\text{C}^{15}\text{N}$]-FAK was able to both autophosphorylate (as distinguished by the presence of pY³⁹⁷ following incubation of [$^{13}\text{C}^{15}\text{N}$]-FAK alone in kinase buffer), and be phosphorylated by Src on multiple Y residues, including activation loop Y⁵⁷⁶ and Y⁵⁷⁷, forming a mixture of unphosphorylated-[$^{13}\text{C}^{15}\text{N}$]-FAK, pY⁵⁷⁶-[$^{13}\text{C}^{15}\text{N}$]-FAK, and pY⁵⁷⁶/Y⁵⁷⁷-[$^{13}\text{C}^{15}\text{N}$]-FAK (data not shown).

LC-MS analysis of FAK activation loop and control segment tryptic peptides

Chromatograms for the activation loop peptides for unphosphorylated-FAK, pY⁵⁷⁶-FAK, and pY⁵⁷⁶/pY⁵⁷⁷-FAK, as well as the control peptide EVGLALR, derived from LC-MRM/MS analysis of an unlabeled peptide/[$^{13}\text{C}^{15}\text{N}$]-FAK standard digest mixture, and their corresponding heavy isotope internal standards are shown in Figure 3. Standard curves for endogenous FAK were generated using the peak area ratio of unlabeled peptide to [$^{13}\text{C}^{15}\text{N}$]-labeled peptide, while standard curves for [$^{13}\text{C}^{15}\text{N}$]-labeled FAK were generated using the peak area ratio of labeled FAK to [$^{13}\text{C}^{15}\text{N}$]-labeled peptide. Standard curves were linear for all peptides in the range of 0.08 to 1 pmol, except for YMEDSTpY⁵⁷⁶pY⁵⁷⁷K, which was analyzed in the range of 0.02 to 0.35 pmol. In addition, linear standard curves to measure the higher amounts of recombinant FAK used in the *in vitro* experiments were prepared in the range of 0.5 to 2 pmol. The standard curves for [$^{13}\text{C}^{15}\text{N}$]-FAK peptides were generated over a range of 2 % to 50 % (where 100 % corresponded to 12 μL of a stock solution containing 0.514 pmol unphosphorylated, 0.404 pmol pY⁵⁷⁶, and 0.118 pmol pY⁵⁷⁶/pY⁵⁷⁷ [$^{13}\text{C}^{15}\text{N}$]-FAK). Typical regression lines for the unlabeled unphosphorylated, mono- (pY⁵⁷⁶), bis-phosphorylated (pY⁵⁷⁶/pY⁵⁷⁷) activation loop peptides, and control segment peptides in the range for measuring endogenous FAK were: $y = 1.00681x + 0.00278966$ ($r^2 = 0.9957$), $y = 1.08214x - 0.0101478$ ($r^2 = 0.9981$), $y = 1.02961x - 0.014805$ ($r^2 = 0.9899$), and $y = 2.673x - 0.193129$ ($r^2 = 0.9990$), respectively (Supplemental Figures 3, 4, 5, and 6). Typical

regression lines for [$^{13}\text{C}^{15}\text{N}$]-FAK unphosphorylated and mono-phosphorylated (pY 576) were $y = 0.00521341x + 0.00630904$ ($r^2 = 0.9966$) and $y = 0.00420133x - 0.00433783$ ($r^2 = 0.9927$), respectively (supplemental Figures 7 and 8).

Following classic standard isotope dilution LC-MS methodology, both endogenous and [$^{13}\text{C}^{15}\text{N}$]-FAK amounts were calculated from regression lines of relevant standard curves based on their response ratio to [$^{13}\text{C}^{15}\text{N}$]-peptide standards.^{17;27} Initial reports on measuring protein phosphorylation using heavy isotope-labeled standard peptides validated that the peptide standards could accurately quantify protein or phosphorylation levels from a sample resolved and digested in gel.²² However, it is important to construct standard curves over the entire concentration that is being studied and it cannot be assumed that a regression line will continue to be linear either below the lowest or above the highest analytical standard. The current method has employed such methodology and so the quantification has been conducted under the most accurate conditions possible. This is a particularly important methodology when measuring *bis*-phosphorylated or very hydrophobic peptides that can readily bind to surfaces.^{18;28} These issues made it necessary to keep the phosphorylated peptides in solutions containing 10 mM EDTA, which allowed the generation of linear standard curves over the range of concentrations required for the cell-based studies. Without such precautions, the amount of pY 576 /pY 577 -FAK present in cell culture would have been extremely inaccurate.

Validation experiments

Recombinant FAK was treated with recombinant Src in the presence of ATP. At various time points, the reaction was stopped by heat inactivation of the kinases and the amount of activation loop unphosphorylated, mono-phosphorylated pY 576 , *bis*-phosphorylated pY 576 /pY 577 , and the control peptide were quantified using heavy isotope-labeled peptide standards. Within the first 15 min, the presence of Src led to maximal conversion of the unphosphorylated FAK activation loop into pY 576 and pY 576 /pY 577 forms (Figure 4A). At the zero time point, 0.998 pmol of unphosphorylated Y 576 /Y 577 and 0.247 pmol pY 576 were detected, while pY 576 /pY 577 was below the detection limit. After a 15 min incubation, 0.060 pmol of unphosphorylated Y 576 /Y 577 , 0.768 pmol pY 576 , and 0.342 pmol pY 576 /pY 577 were present. In contrast to changes in the level of phosphorylation of the FAK activation loop region, the sum total of all activation loop forms and the control segment peptide remained unaltered during this time (Figure 4B). Furthermore, the sum of the activation loop forms averaged over all reaction time points was in agreement with the amount of control peptide averaged over all time points, with 1.108 (± 0.048) pmol total activation loop forms compared to 1.051 (± 0.040) pmol control peptide (Figure 4C).

Another *in vitro* kinase reaction was conducted, allowing recombinant Src to phosphorylate recombinant FAK. This reaction was stopped by heating the mixture to 70 °C for 10. A portion of this reaction mixture was added to a dish, or split into two dishes of lysing FAK-/- MEFs. [$^{13}\text{C}^{15}\text{N}$]-FAK was also added to the lysing cell monolayers. Total FAK was immunoprecipitated and the unlabelled recombinant FAK and [$^{13}\text{C}^{15}\text{N}$]-FAK activation loop peptides and the control peptide were quantified (Figure 2). In parallel, a portion of the kinase reaction was mixed with [$^{13}\text{C}^{15}\text{N}$]-FAK in a vial, and the activation loop peptides and control peptide were quantified. The amounts of unphosphorylated pY 576 , and pY 576 /pY 577 FAK present in the kinase reaction mixture before the immunoprecipitation were 0.707 (± 0.029), 1.893 (± 0.025), and 1.080 (± 0.021) pmol, respectively. Following immunoprecipitation, the amounts quantified were 0.160 (± 0.011) pmol unphosphorylated, 0.338 (± 0.018) pmol pY 576 , and 0.187 (± 0.011) pmol pY 576 /pY 577 FAK. The immunoprecipitation recovery based on [$^{13}\text{C}^{15}\text{N}$]-FAK was 24.5 % for unphosphorylated -FAK and 15.1 % for pY 576 -FAK. Applying these correction values for each sample to the initial immunoprecipitated amounts resulted in adjusted amounts of 0.669 (± 0.030) pmol for unphosphorylated -FAK, 2.123 ($\pm$

0.191) pmol for pY⁵⁷⁶-FAK, and 1.221 ($\pm$ 0.063) pmol for pY⁵⁷⁶/pY⁵⁷⁷-FAK (Figure 5A). The total amount of activation loop peptides (corrected for losses during immunoprecipitation) was 4.025 ($\pm$ 0.298) pmol and the amount control peptide was 3.891 ($\pm$ 0.278) pmol (Figure 5B). The total FAK amount based on the control peptide was therefore 3.891 ($\pm$ 0.278) pmol (i.e. 486 ng FAK/1.65 mg total protein (single dish background) and 486 ng FAK/3.30 mg total protein (double dish background)).

The two validation experiments were performed in order to show that (a) the control peptide tracked with the sum of activation loop forms and (b) that the *in vitro* produced [¹³C¹⁵N]-FAK protein was immunoprecipitated at an equivalent rate to cellular endogenous FAK. We have previously reported on the relative quantification of all phosphotyrosine sites on FAK following Src treatment *in vitro* using LC-MRM/MS.¹⁹ In a similar experimental workflow, samples were collected at different time points during a 120 min reaction of Src with recombinant FAK. These samples were spiked with heavy isotope-labeled peptide standards and digested with trypsin. The result of this proof-of-principle experiment showed that the sum of unphosphorylated -FAK, pY⁵⁷⁶-FAK, and pY⁵⁷⁶/pY⁵⁷⁷-FAK was consistent with the calculated molar amount of control peptide. It should be noted that at time points greater than 30 min, a disappearance of a small amount of FAK protein was observed. Although the amount of all forms of the activation loop region can be seen to decrease at later time points, the amount of control region was reduced equivalently. It is possible that at these time points FAK degraded or was lost due to nonspecific absorbance to the incubation tube. This validated that the prepared standard curves were accurate and demonstrated that it was possible to quantify very small changes in the level of FAK phosphorylation.

The second aspect of validation involved quantification of the Src-treated recombinant FAK before immunoprecipitation and after immunoprecipitation from either a single (low background) or two (high background) confluent dishes of lysing FAK^{-/-} MEFs. These data showed that the amount of immunoprecipitated FAK, against the background of either one or two plates, was identical to the expected total amount of recombinant FAK present. More importantly, the calculated immunoprecipitation recovery based on recovered [¹³C¹⁵N]-FAK correctly adjusted for any losses that had occurred. Interestingly, the immunoprecipitation recovery differed depending on the presence of Y⁵⁷⁶ and Y⁵⁷⁷ phosphorylation, which consistently showed the unphosphorylated form was immunoprecipitated more efficiently. The immunogen designed by the manufacturer to generate the antibody used for the immunoprecipitation (anti-FAK clone 4.47) was the first 423 amino acids of FAK fused to GST. Therefore, although Y⁵⁷⁶ and Y⁵⁷⁷ phosphorylation would not directly alter the epitope recognized by the antibody, a structural alteration affecting the interaction of these residues and the epitope is certainly possible. This highlights the importance of using an appropriately labeled protein to monitor the recovery.

LC-MRM/MS analysis of FAK activation loop phosphorylation in MEFs

Confluent plates of MEFs²⁵ were lysed in the presence of [¹³C¹⁵N]-FAK and total FAK was immunoprecipitated. [¹³C¹⁵N]-Peptide standards were added to the immunoprecipitate elution and the mixture was digested with trypsin. Tryptic digests were analyzed by LC-MRM/MS to quantify both endogenous FAK as well as [¹³C¹⁵N]-FAK. A typical chromatogram for digested endogenous and [¹³C¹⁵N]-FAK immunoprecipitated from the MEFs and their internal standards is shown in Figure 6. Endogenous and [¹³C¹⁵N]-FAK peptides were quantified using their respective calibration curves. Endogenous FAK peptide levels were then corrected using the calculated immunoprecipitation recovery value (using the [¹³C¹⁵N]-FAK standard) and normalized to total FAK (using the amount unmodified control peptide) as demonstrated during validation experiments. MEFs contained 0.654 ($\pm$ 0.034) pmol of unphosphorylated -FAK, 0.331 ($\pm$ 0.052) pmol of pY⁵⁷⁶-FAK, and 0.059 ($\pm$ 0.010) pmol of pY⁵⁷⁶/pY⁵⁷⁷-FAK. The total

amount of activation loop peptides normalized to the control peptide for MEFs in the basal state was $1.044 (\pm 0.087)$ pmol (Figure 7). This value was determined using calculated immunoprecipitation recoveries of 12.0 % unphosphorylated and 8.5 % phosphorylated FAK. The average total FAK amount in the normal MEFs (corrected for losses during immunoprecipitation) was 5.15 pmol/plate or 216.8 ng FAK/mg of total protein. Relative amounts of the individual forms were $65.4 (\pm 3.4)$ % of unphosphorylated FAK, $33.1 (\pm 5.2)$ % of pY⁵⁷⁶-FAK, and $5.9 (\pm 1.0)$ % of pY⁵⁷⁶/pY⁵⁷⁷-FAK as shown in Figure 7.

Oncogenic Y⁵²⁹FSrc is constitutively activated and causes FAK phosphorylation because it lacks regulation by C-terminal tail phosphorylation.²⁵ This causes significant changes to MEF morphology. The calculated immunoprecipitation recoveries of unphosphorylated FAK and phosphorylated-FAK in Y⁵²⁹FSrc-expressing MEFs were 25 % and 14 %, respectively. The average total FAK amount in the Y⁵²⁹FSrc expressing MEFs (corrected for losses during immunoprecipitation) was 1.5 pmol/plate or 75.9 ng FAK/mg of total protein. Therefore, immunoprecipitation efficiency increased significantly for both FAK and phosphorylated FAK when cells expressed lower amounts of total FAK. In contrast (as described above) the amount of total protein did not affect the immunoprecipitation recovery if the total FAK content was kept constant.

CONCLUSIONS

Protein isolation using methodology such as immunoprecipitation prior to protease digestion and LC-MS analysis is often employed in proteomic investigations. However, the internal standards typically utilized to quantify proteins (peptide standards) are generally added post-isolation so that they do not account for protein losses during sample workup. Furthermore, it is generally assumed that unmodified proteins and proteins with PTMs are isolated with equal efficiency by immunoprecipitation. In order to control for FAK recovery, to ensure that the stoichiometry of the different molecular forms of FAK's activation loop were not altered during, or as a result of, immunoprecipitation, and to correct for any FAK proteins lost between cell harvesting and trypsin digestion, full-length heavy isotope-labeled and phosphorylated FAK proteins were prepared as internal standards. They were then employed in stable isotope dilution LC-MRM/MS assays.

Relevant labeled FAK proteins were synthesized *in vitro* using a cell free transcription and translation system supplied with [¹³C₆¹⁵N₄]-arginine, [¹³C₉]-tyrosine, [¹³C₆¹⁵N₂]-lysine, and [¹³C₆¹⁵N]-leucine in a similar manner to other published techniques.^{21;24} Use of the *in vitro* system efficiently produced sufficient amounts of protein for use as an internal standard in extensive cell based assays. One drawback to this technique is that, unlike endogenous translation in the endoplasmic reticulum in which chaperone proteins ensure proper protein folding, *in vitro* synthesis can result in the expression of a non-functional and misfolded protein. In order to overcome this obstacle, the synthetic labeled FAK was denatured and allowed to refold during buffer exchange into an oxidizing buffer; which is similar to an approach that has been reported previously.²⁹ The re-folding protocol resulted in generation of a FAK protein that was capable of both autophosphorylation on Y³⁹⁷ and trans-phosphorylation by Src on other known sites of tyrosine phosphorylation, including the activation loop Y⁵⁷⁶ and Y⁵⁷⁷ residues (Figure 1).

A tryptic peptide from FAK (E⁹⁵⁶VGLALR⁹⁶²) that was unable to undergo PTM was quantified in each experiment. This was an important control because the FAK activation loop tryptic peptide YMEDSTY⁵⁷⁶Y⁵⁷⁷K, could conceivably exist in thirty-five different forms through phosphorylation of Y⁵⁷¹, S⁵⁷⁴, and T⁵⁸⁵ as well as through oxidation of M⁵⁷². However, we were able to demonstrate that the sum of unphosphorylated Y⁵⁷⁶/Y⁵⁷⁷, pY⁵⁷⁶, and pY⁵⁷⁶/pY⁵⁷⁷ corresponded to the molar amount of control peptide isolated from a kinase

reaction conducted *in vitro* (Figures 4 and 5) as well as from a cell-based system (Figure 7). These experiments confirmed that other PTMs on the activation loop domain were insignificant. Absolute quantification was conducted using stable isotope labeled peptide standards with four endogenous amino acid overhangs at the trypsin digestion sites of both the amino and carboxy terminus. It was essential that standard curves were prepared over the expected range of peptide concentrations in order to ensure that selective losses of phosphorylated peptides did not occur.

Interestingly, mono-phosphorylation of Y⁵⁷⁷ was not detected in the MEFs (Figure 7). This is in keeping with the concept that phosphorylation on Y⁵⁷⁶ results in a conformational change in FAK so that Y⁵⁷⁷ is then phosphorylated to give a *bis*-phosphorylated protein. Recent structural studies have revealed an autoinhibitory mechanism whereby FAK's N-terminal FERM domain interacts with the face of the kinase domain to effectively shield its accessibility to substrate.³⁰ This autoinhibitory interaction is critically dependant on Y⁵⁷⁷, which when phosphorylated by Src, results in the relief of the FERM-kinase domain interaction allowing FAK to form an open and active conformation capable of phosphorylating other substrates. The *bis*-phosphorylated form corresponded to 5.9 ± 1.0 % of total FAK in normal MEFs (Figure 7). There were significant amounts of unphosphorylated (65.4 ± 3.4 %) and mono-phosphorylated pY⁵⁷⁶ (33.1 ± 5.2 %) inactive forms of FAK (Figure 7) illustrating the dynamic balance that exists between kinases (such as Src) and phosphatases to modulate the activity of cellular FAK in normal MEFs.

Overall, our new approach ensures rigorous quantification of FAK phosphorylation and provides a way to determine if an unexpected PTM has occurred. Immunoprecipitation recovery from cells was consistently more efficient for unphosphorylated-FAK when compared with phosphorylated-FAK. Furthermore, the absolute recovery of both unphosphorylated and phosphorylated forms of FAK increased as the amount of total cellular FAK decreased. The increased immunoprecipitation efficiency did not result from different amounts of total (non-FAK) cellular protein expression. These findings further highlight the importance of using labeled proteins in quantitative determinations to correct for differential recoveries during the isolation procedure. Two previous studies have reported the use of heavy isotope labeled protein internal standards for protein quantification.^{20,21} However, these methods are not extendable to the quantification of protein post-translational modifications. Hence, they are not appropriate for the study of cellular signal transduction pathways. In the present study, two different phosphorylated forms of the protein together with the non-phosphorylated form were quantified both *in vitro* and in cell culture systems after immunoprecipitation. Importantly, we were able to determine the recovery of each molecular form after immunoprecipitation and to show that there were no unexpected sites of post-translational modifications on the activation loop domain. This has made it possible, for the first time, to accurately quantify a multiply phosphorylated protein involved in cell signaling through the action of kinases and phosphatases. Availability of this stable isotope dilution LC-MRM/MS methodology will have particular utility for future biomarker studies of kinase inhibiting anti-cancer drugs such as AZD0530,³¹ and for quantitative proteomic investigations that examine kinase- and phosphatase-mediated cellular signal transduction pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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1-  MAAAYLDPNL NHTPNSSTKT HLTGMERSP GAMERVLKVF HYFESNSEPT TWASIIRHGD
61-  ATDVRGIIQK IVDSHKVKHV ACYGFRLSHL RSEEVHVLHV DMGVSSVREK YELAHPPPEEW
121-  KYELRIRYLP KGFLNQFTED KPTLNFFYQQ VKSDYMLEIA DQVDQEQALK LGCLEIRRSY
181-  WEMRGNAL EK KSNYEVLKED VGLKRFFPKS LLDVSKAKTL RKLIIQQTFRQ FANLNRRESI
241-  LKFFEILSPV YRFDKECFKC ALGSSWISV ELAIGPEEGI SYLTDKGCNP THLADFTQVQ
301-  TIQYNSSEDK DRKGMLQLKI AGAPEPLTVT APSLTIAENM ADLIDGYCRL VNGTSQSFI
361-  RPQKEGERAL PSIPKLANSE KQGMRTHAVS VSETDDY397 AEI IDEEDY407 TMP STRDYEQRE
421-  RIELGRGIGE GQFGDVHQGI YMSPENPALA VAIKCKNCT SDVREKFLQ EALTMRFQDH
481-  HIVKLIGVI TENPVWIIIE LCTLGELRSF LQVRKYSIDL ASLILYAYQL STALAYLESK RFVHRDIAAR
541-  NVLVSSNDCV KLGDFGLSRV570 MEDSTY576Y577K578 AS KGKLPKWMA PESINFRRT
601-  SASDVWMFGV CMWEILMHGV KPFQGVKNND VIGRIENGER LPMPPNCPPT LYSLMTKCWA
661-  YDPSRRPRFT ELKAQLSTIL EEEKAQQEER MRMESRRQAT VSWDSGGSDE APPKPSRPGY
721-  PPSRSSEGFY PSPQHVMVQTN HYQVSGYPGS HGITAMAGSI YPGQASLLDQ TDSWNHRPQE
781-  IAMWQPNVED STVLDLRGIG QVLPTHLMEE RLIRQQQEME EDQRWLEKEE RFLKPDVRLS
841-  RGSIDREDGS LQGPIGNQHI Y861QPVGKPDPA APPKKPPRPG APGHLGSLAS LSSPADSYNE
901-  GVKLPQDEIS PPPTANLDRS NDKVY925ENVTG LVKAVIEMSS KIQAPPEEY VPMVKE956VGLA
961-  LR962TLLATVDE TIPLLPASTH REIEMAQKLL NSDLGELINK MKLAQQYVMT SLQQEYKKQM
1021- LTAHALAVD AKNLLDVIDQ ARLKMLGQTR PH

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Figure 1.

FAK amino acid sequence. The amino acid sequence of FAK is shown, where residues shown in bold correspond to the tryptic peptide from the kinase activation loop domain (Y⁵⁷⁰ to K⁵⁷⁸), the control tryptic peptide (E⁹⁵⁶ to R⁹⁶²), and important sites of tyrosine phosphorylation (Y³⁹⁷, Y⁴⁰⁷, Y⁵⁷⁶, Y⁵⁷⁷, Y⁸⁶¹, and Y⁹²⁵).

Figure 2.

Experimental workflow for absolute quantification of FAK activation loop and control peptides following protein immunoprecipitation. A full length isotopically-labeled and phosphorylated FAK protein ($[^{13}\text{C}^{15}\text{N}]$ -FAK) was spiked onto cell monolayers during harvesting. Endogenous and $[^{13}\text{C}^{15}\text{N}]$ -FAK were immunoprecipitated. Following elution from the immune-complex, the FAK mixture was spiked with $[^{13}\text{C}^{15}\text{N}]$ -labeled peptide standards correlating to sites of activation loop phosphorylation at Y⁵⁷⁶ and Y⁵⁷⁷ in unphosphorylated, mono- (pY⁵⁷⁶), and bis-phosphorylated (pY⁵⁷⁶/pY⁵⁷⁷) forms. In addition, a $[^{13}\text{C}^{15}\text{N}]$ -labeled peptide correlating to a separate unmodified segment of FAK was included as a control. Trypsin digestion of the mixture resulted in the hydrolysis of endogenous FAK, $[^{13}\text{C}^{15}\text{N}]$ -FAK, and $[^{13}\text{C}^{15}\text{N}]$ -labeled peptide standards. LC-MRM/MS analysis of the resultant twelve tryptic peptides was then conducted to quantify the amount of endogenous and $[^{13}\text{C}^{15}\text{N}]$ -FAK, allowing for determined amounts of activation loop peptides to be corrected using the calculated immunoprecipitation value (using the $[^{13}\text{C}^{15}\text{N}]$ -FAK standard) and normalized to the amount total FAK (using the unmodified control segment peptide).

Figure 3.

LC-MRM/MS analysis of an unlabeled peptide/ [¹³C¹⁵N]-FAK standard digest mixture, and their corresponding heavy isotope-labeled internal standards. MRM transitions are shown in Table 1.

Figure 4.

Quantitative analysis of FAK activation loop Y⁵⁷⁶ and Y⁵⁷⁷ phosphorylation during an *in vitro* kinase reaction with recombinant normal Src: control peptide validation. Recombinant FAK (258.8 nM) and Src (12.8 nM) were incubated in the presence of ATP (1 mM) at 37 °C. At various time points over a 120 min reaction, a portion of the reaction was removed, spiked with heavy isotope-labeled peptide standards, and digested with trypsin. LC-MRM/MS was used to quantify the amount of activation loop peptides in the unphosphorylated (◇), mono-phosphorylated (pY⁵⁷⁶, □), and bis-phosphorylated (pY⁵⁷⁶/pY⁵⁷⁷, △) forms (A). In addition to measuring the amount of activation loop peptides, a separate unmodified segment of FAK was measured as a control. The amount of unphosphorylated Y⁵⁷⁶/Y⁵⁷⁷ (□), mono-pY⁵⁷⁶ (■), bis-phosphorylated pY⁵⁷⁶/pY⁵⁷⁷ (▨) activation loop peptides, the sum amount of activation loop peptide (■), and the amount of control peptide (■) at three representative time points are shown in (B). The sum of activation loop peptides and the amount of control peptide averaged over all time points are shown in (C), where error bars are + SEM for eleven time points.

Figure 5.

Quantitative analysis of FAK activation loop Y⁵⁷⁶ and Y⁵⁷⁷ phosphorylation before and following immunoprecipitation from FAK ^{-/-} MEF lysate background: immunoprecipitation validation. A portion of an *in vitro* Src treated recombinant FAK reaction was quantified before immunoprecipitation and following immunoprecipitation from FAK ^{-/-} MEFs. The initial amount of activation loop peptides following immunoprecipitation [IP(raw)] were corrected [IP(corrected)] using the calculated immunoprecipitation recovery value based on [¹³C¹⁵N]-FAK. Shown in (A) are the amounts of unphosphorylated Y⁵⁷⁶/Y⁵⁷⁷ (□), mono- pY⁵⁷⁶ (▒), and bis-phosphorylated pY⁵⁷⁶/pY⁵⁷⁷ (▨) activation loop peptides. The sum amount of

activation loop peptides (■) compared to the amount of control segment peptide (□) post-immunoprecipitation (corrected) are shown in (B).

Figure 6. LC-MRM/MS analysis of activation loop and control peptides from digested endogenous and [¹³C¹⁵N]-FAK immunoprecipitated from MEFs expressing normal Src. MRM transitions are shown in Table 1.

Figure 7.

Quantification of FAK activation loop Y⁵⁷⁶ and Y⁵⁷⁷ in MEFs expressing normal Src. Shown are amounts of unphosphorylated -Y⁵⁷⁶/Y⁵⁷⁷-FAK (□), pY⁵⁷⁶-FAK (▤), and pY⁵⁷⁶/pY⁵⁷⁷-FAK (▨) activation loop peptides, and the total amount of activation loop peptides (■) from a FAK trypsin digest after immunoprecipitation from confluent monolayers of MEFs expressing normal Src or oncogenic Y⁵²⁹FSrc following pervanadate inhibition of global tyrosine phosphatases. Data are amounts of each peptide corrected using the calculated immunoprecipitation value and normalized to the amount total FAK. Amounts represent the average of three separate experiments, where error bars are + SEM ($n = 3$).

Table 1

MRM transitions for the analysis of FAK activation loop phosphorylation status. [$^{13}\text{C}^{15}\text{N}$]-labeled amino acids are indicated with an asterisk.

Parent Sequence	Parent m/z	Product	Product m/z	Source
YMEDSTYYK	600.25 ²⁺	y7	905.4	Endogenous-FAK
YMEDSTYY*K	604.25 ²⁺	y7	913.4	[$^{13}\text{C}^{15}\text{N}$]-peptide std
*YMEDST*Y*Y*K	617.25 ²⁺	y7	930.4	[$^{13}\text{C}^{15}\text{N}$]-FAK
YMEDSTpYYK	640.25 ²⁺	y7 and y7 (- H ₂ O)	985.35 and 967.35	Endogenous-FAK
YMEDSTpYY*K	644.25 ²⁺	y7 and y7 (- H ₂ O)	993.36 and 975.36	[$^{13}\text{C}^{15}\text{N}$]-peptide std
*YMEDSTp*Y*Y*K	657.25 ²⁺	y7 and y7 (-H ₂ O)	1010.36 and 992.36	[$^{13}\text{C}^{15}\text{N}$]-FAK
YMEDSTpYpYK	680.25 ²⁺	y7 (-H ₂ O)	1047.31	Endogenous-FAK
YMEDSTpYpY*K	684.25 ²⁺	y7 (-H ₂ O)	1055.32	[$^{13}\text{C}^{15}\text{N}$]-peptide std
*YMEDSTp*Y*pY *K	697.25 ²⁺	y7 (-H ₂ O)	1072.32	[$^{13}\text{C}^{15}\text{N}$]-FAK
EVGLALR	379.23 ²⁺	y3	359.24	Endogenous-FAK
E*VGLALR	382.23 ²⁺	y3	359.24	[$^{13}\text{C}^{15}\text{N}$]-peptide std
EVG*LA*L*R	391.23 ²⁺	y3	376.24	[$^{13}\text{C}^{15}\text{N}$]-FAK