Targeted Identification and Quantitative Monitoring of Phosphorylation Changes of Focal Adhesion Kinase Protein

*MIDAS™ Workflow for Phosphorylation Studies using the 4000 Q TRAP® System*

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The analysis of phosphorylation sites on proteins relies heavily on the use of immunologically-based methodology. However, the mass spectrometry (MS)-based methodologies are increasingly being used because of the additional specificity that can be attained. Determining the temporal sequence of phosphorylation at multiple sites within a single protein still remains a significant analytical challenge but is key to understanding the biological process. For example, focal adhesion kinase (FAK), a protein with 1052 amino acid residues, has thirty-eight Y, eighty-one S, and fifty-one T residues, all of which can potentially be phosphorylated. Phosphorylation has been detected by molecular and biochemical techniques on only eleven of the S and six of the Y residues.¹⁻⁷ Sites of phosphorylation that are thought to be the most important in controlling signal transduction pathways are shown in Figure 1.

Focal adhesions are composed of integrin adhesion receptors (transmembrane heterodimers) and a focal complex (a multi-protein plaque associated with the cytoplasmic tail of integrins). Phosphorylation of this multi-protein plaque is dependent upon non-receptor protein tyrosine kinases (PTKs), two of which, c-Src and focal adhesion kinase (FAK), play a dominant role. These kinases are key regulators of integrin signaling at focal adhesions as well as a number of growth factor signaling pathways. In this way, Src and FAK activities integrate multiple signaling events guiding cell growth, migration, and differentiation.

In order to monitor specific phosphorylation events quantitatively on FAK, an MS-based approach was utilized. The Multiple Reaction Monitoring (MRM) initiated detection and sequencing strategy (the MIDAS™ Workflow) was used to identify and monitor twenty-nine peptides resulting from the tryptic digestion of FAK that contained all of the known sites of phosphorylation together with a number of other potential phosphorylation sites.⁸⁻¹⁰ This technique was able to qualitatively differentiate between autocatalytic and Src-induced phosphorylation events.¹¹ In conjunction with in vitro kinase assays, the current model of FAK autophosphorylation and Src-induced phosphorylation was tested using this LC-MRM technology.

Figure 1. Schematic of FAK protein. Phosphorylation sites indicated have previously been reported and experimentally verified to play important roles in regulating FAK kinase activity and guiding protein-protein interaction.
Materials and Methods

**In Vitro kinase reaction:** Human FAK (NP_722560) and human c-Src were incubated alone or together in kinase buffer with and without ATP. The reactions were incubated with or without c-Src at either 4°C or 37°C for 30 or 60 minutes. After 30 or 60 minutes, the reactions were immediately stopped.

**Sample Preparation:** The reaction mixture was reduced, alkylated, and digested in solution using trypsin. Following digestion at 37°C overnight, the sample volume was reduced by evaporation at room temperature in a vacuum concentrator/centrifugal evaporator. The sample was then mixed 1:1 with H2O and stored until analyzed.

**Chromatography:** Using a Tempo™ nanoLC system, the sample was loaded onto a trap column (PepMap™100, 300 µm x 5 mm), washed for 20 min, then eluted onto a C18 reversed phase column (PepMap™ 75µm x 150mm). Peptides were eluted at a flow rate of 200 nL/min.

**Mass Spectrometry:** All MS was conducted using a 4000 QTRAP® system operated in the positive mode using the Nanospray® Source and heated interface. A MIDAS™ Workflow method was developed for phosphopeptides determined from both full scan MS based experiments and phosphotyrosine (pY) precursor ion scanning experiments. In addition, MRM transitions were included to monitor a peptide in both its unmodified and phosphorylated forms. All MIDAS workflow methods utilized information dependent acquisition where the MRM survey scan triggered an enhanced resolution (ER) scan on the two most intense MRMs above an intensity threshold (1000 cps). These ER scans then triggered enhanced product ion scans (EPI) where parent ions were subjected to collision-induced dissociation using rolling collision energy (Figure 2). MRM design consisted of setting the Q1 m/z to the most intense parent ion charge state and the Q3 m/z to the most intense product ion. In all, twenty-nine MRM transitions were monitored. When determining phosphorylation changes in various conditions, samples were analyzed in duplicate.

**Data Processing:** MS/MS analysis and database searching was performed using ProteinPilot™ software and the SwissProt FASTA file (uniprot_sprot20051220.fasta). All ProteinPilot Software database searches were performed using the Paragon™ database search algorithm in Thorough mode and the Pro Group™ Algorithm.
Phosphotyrosine-Peptide Identification

LC/MS/MS of Focal Adhesion Kinase tryptic digest identified the protein along with a number of phosphorylated and non-phosphorylated peptides. Precursor ion scanning for the immonium ion of pY (m/z 216, Scheme 1) or the PO3- ion (m/z – 79) identified a number of phosphorylated peptides which were not detected in the non-targeted full scan MS approach. In total, eleven tryptic peptides to FAK were found to be phosphorylated, containing 17 different sites of phosphorylation.

All phosphorylated sites that had been identified, as well as a number of hypothetical phosphorylated sites were used to create the LC-MRM method highlighted in Figure 3.

Scheme 1. Precursor Scanning for the Immonium Ion of pY. The pY immonium ion is generated by a combination of α- and γ-type cleavage of a pY containing peptide, producing a diagnostic ion m/z 216.

Figure 3. (A) Sequences of 11 FAK Tryptic Peptides Found to be Phosphorylated. The phosphorylated S, T, and Y residues are shown in red, with the novel sites of phosphorylation identified here shown in bold. (B) MRM transitions monitored for each phosphorylation site and (C) the LC MRM chromatogram for a typical Focal Adhesion Kinase tryptic digest.
Characterizing Phosphorylation of Y861

The main site of Src phosphorylation of FAK, toward the C terminus of its kinase domain, occurs on Y861 (Figure 1).12

Trypsin digestion of FAK yielded the large peptide G842S843DREDGS850LQGPIGNQHIY861QPVGKPDPAAPPK874 containing Y861. This peptide was complex and was observed as the unmodified, mono-phosphorylated, bis-phosphorylated, and tris-phosphorylated forms. As a result of this complexity, the MIDAS™ workflow on the 4000 QTRAP® system with LC resolution was essential for determining the sites of phosphorylation (Figure 5).

MIDAS workflow identified two mono-phosphorylated forms with retention time values of 19.1 and 21.1 min, containing pY861 and pS843, respectively. Two bis-phosphorylated forms, with retention time values of 17.9 and 20.3 min, were identified as containing pS843pS850 and pS843pY861, respectively. Finally, a single tris-phosphorylated form of the peptide with a retention time of 17.2 min was identified as containing pS843pS850pY861 as well as the un-modified peptide with a retention time at 20.1 min (Figure 4).

No auto-catalytic activity toward Y861 was observed by quantitative LC-MRM when the basal state (Figure 4) was compared with the addition of ATP at either 4°C (Figure 6A) or 37°C (Figure 6B). Similarly, essentially no phosphorylation was observed when Src and ATP were added to FAK at 4°C (Figure 6C). However, there was a dramatic increase in the amounts of pY861- and pS843pS850pY861-containing peptides as shown by the large increase in the specific MRM peak area, together with a decrease in the unmodified peptide, the mono-phosphorylated peptide containing pS843, and the bis-phosphorylated peptide containing pS843pS850 when the reaction was conducted at 37 °C (Figure 6D).

Figure 4. Analysis of FAK phosphorylation on Y861 by nanospray LC-MRM/MS. Unmodified (red), mono-phosphorylated (blue), bis-phosphorylated (black), and tris-phosphorylated (green) forms of FAK tryptic peptide G842-K874 in the basal state.

Figure 5. Identifying Specific Phosphorylation Sites. The MIDAS workflow was used to identify the specific sites of phosphorylation for the G842S843DREDGS850LQGPIGNQHIY861QPVGKPDPAAPPK peptide. Shown is the singly phosphorylated peptide at Y861. 5 specific phosphorylation forms of this peptide were identified (Figure 4).
Conclusions

FAK phosphorylation by Src has been the subject of intense experimental scrutiny. A current model of FAK-Src interaction has been developed from many lines of experimentation, including in vitro and in vivo kinase assays using endogenous, as well as mutant forms of both FAK and Src.

We have developed an LC-MRM based method that made it possible to examine seventeen potential S, T, or Y phosphorylation sites on eleven tryptic peptides from FAK and to assess the role that Src plays in phosphorylating these sites. Our new method has identified six novel phosphorylation sites and allowed detailed mechanistic studies to be conducted on four additional sites. We have demonstrated that Src is able to phosphorylate Y861 at 37 °C, regardless of the phosphorylated S residues that are present in the FAK. Two additional tryptic peptides, T386-HAVS389-VS392-ET394-DDY397-AEIIDEEDTY407-TMPSTR413 and Y570-MEDSTY576-Y577-K578 were also monitored and shown to be phosphorylated at different sites under the conditions studied. Finally, the newly identified S and T phosphorylated residues on FAK were not altered by interactions with Src in keeping with its known tyrosine kinase activity. The characterization of these phosphorylated residues suggests that there are as yet unknown S and T kinases that are involved in FAK-mediated cellular signal transduction. Additional studies will be required to identify these kinases in cellular systems. The ability to readily quantitatively monitor the phosphorylation sites by LC-MRM will greatly facilitate such future studies and is key to the understanding of underlying biology of phosphorylation.

Figure 6. Analysis of FAK phosphorylation on Y861 by nanospray LC-MRM/MS. MRM analysis of unmodified (red), mono-phosphorylated (blue), bis-phosphorylated (black), and tris-phosphorylated (green) forms of FAK tryptic peptide G342-K874. (A) FAK/ATP/4°C (B) FAK/ATP/37°C (C) FAK/Src/ATP/4°C (D) FAK/Src/ATP/37°C.
References