

Conformation-Driven Phase Separation in the Linker Domain of Focal Adhesion Kinases

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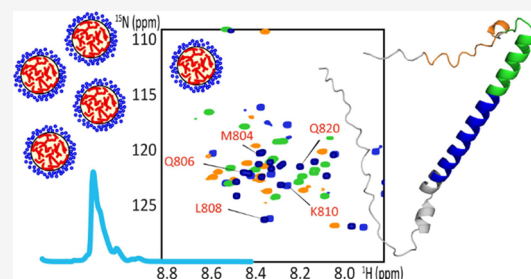
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ABSTRACT: Protein tyrosine kinase 2 (Pyk2), also known as focal adhesion kinase 2, and focal adhesion kinase 1 (Fak1) are two related nonreceptor tyrosine kinases (hereafter referred to as FAKs). Here, we focused on characterizing a linker region of the FAK proteins (hereafter referred to as FAK KFL for Kinase FAT Linker), which in the case of Pyk2 has previously been shown to play a functional role in calcium sensing through its interaction with calmodulin. Using structural nuclear magnetic resonance spectroscopy, we provide chemical shift assignments for the FAK KFLs, defining their conformational properties. Analysis of the FAK KFL conformations revealed their predominantly disordered nature, except for well-defined segments with a significant tendency to form α -helices, which were modeled to form homodimeric interfaces. In addition, we showed that the FAK KFL segments form condensates in vitro under high crowding conditions. By directly comparing the conformational properties of the Pyk2 and Fak1 KFL domains and providing structural data, this study provides valuable insights into the structural basis of FAK KFL interactions. Furthermore, the results show that disordered segments in proteins within the focal adhesion complex undergo phase separation, a process of potential biological significance due to protein clustering.



INTRODUCTION

Protein tyrosine kinase 2 (Pyk2), also known as focal adhesion kinase 2, and focal adhesion kinase 1 (Fak1) are two related nonreceptor tyrosine kinases (hereafter named FAKs) that contribute to the phosphorylation of downstream substrates to relay cellular signals.¹ While Fak1 is ubiquitous, Pyk2 is primarily found in neurons. Both proteins, beyond their kinase activities, function as scaffolding proteins, actively contributing to the formation of signaling complexes, particularly the focal adhesion complex and the NMDA receptor complex. In non-neuronal cells, Fak1 functions as a sensor of extracellular stimuli including the interactions of cells with the extracellular matrix, responding to β -integrin engagement, and subsequently contributing to cell motility and adhesion-mediated cell survival.² Pyk2, on the other hand, has the unique ability to respond to calcium signaling³ and plays an important role in synaptic plasticity both in physiological and pathological contexts.^{2,4–7} Indeed, Pyk2 has been shown to modulate both amyloid- β and tau-induced neurodegeneration in Alzheimer's disease.^{7,8}

The structures of Pyk2 and Fak1 domains have been previously characterized and both FAKs share a common architecture consisting of three consecutive globular domains: a FERM (4,1-ezrin-radixin-moesin) domain at the N-terminus, a central kinase domain and a FAT (Focal Adhesion Targeting) domain at the C-terminus (Figure 1).^{9,10} In both

proteins, the kinase and FAT domains are separated from each other by an extended proline-rich intrinsically disordered linker region, termed the KFL for Kinase Fat Linker,¹¹ which comprises 176 residues in the case of Pyk2 (Pyk2-KFL_{693–868}) and 222 residues in the case of Fak1 (Fak1-KFL_{691–913}) (Figure 1). While the structural and functional properties of the remaining FAK domains are relatively well understood, much less is known about the KFL domains. Previous studies focusing on shorter segments of these KFL domains (Pyk2-KFL_{728–839} and Fak1-KFL_{776–841}) revealed their intrinsically disordered nature (except for a helix-forming segment present in both fragments) and uncovered the formation of fuzzy homodimers which is enhanced by calmodulin binding to Pyk2, suggesting a molecular mechanism responsible for the calcium-sensing activity of Pyk2.¹¹

Despite these significant advances, a comprehensive structural study of the complete disordered domains of KFLs is still lacking, as are investigations of their potential role in cellular processes, such as protein clustering via phase

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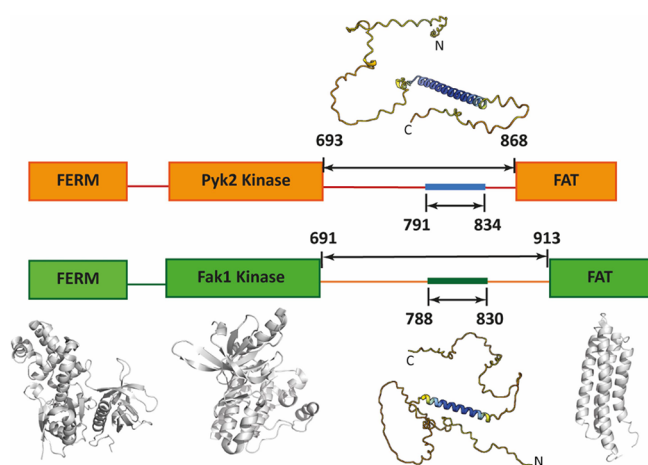


Figure 1. Scheme of the domain organization of Pyk2 and Fak1. The KFL domain corresponds to the linker between the kinase domain and the FAT domain. Sequence conservation of the Pyk2-KFL_{693–868} and Fak1-KFL_{691–913} is low, with only 20.2% identity (33.2% similarity), while their length also varies (176 residues for Pyk2-KFL_{693–868} versus 222 residues for Fak1-KFL_{691–913}). Below the scheme are ribbon representations of the three-dimensional structures of the globular domains of Fak1 (FERM domain: PDB 4CYE, Fak1 kinase domain: PDB 2JKK, FAT domain: PDB 1KTM). A cartoon representation of one representative AlphaFold predicted structure of Pyk2-KFL_{693–868} and Fak1-KFL_{691–913}, color coded according to the PAE (prediction alignment error) from blue to red, is shown. The lower the PAE value, the more credible the structure. Residues at the N and C-termini are annotated.

separation, which may contribute to the formation of signaling complexes. Indeed, disordered polypeptides often have the ability to undergo phase separation due to multivalent weak intermolecular interactions.^{12,13} Of particular relevance to this possibility is recent evidence suggesting that phase separation of focal adhesion proteins may contribute to the formation and maturation of focal adhesions. In addition, Fak1 has been shown to undergo phase separation under physiological conditions, facilitating the clustering of integrins in lipid membranes.^{14–17}

In this study, we build on previous research by elucidating the conformation of the entire FAK KFL regions of Pyk2 (Pyk2-KFL_{693–868}) and Fak1 (Fak1-KFL_{691–913}) using NMR data. We have assigned NMR signals, providing critical insights for future investigation of FAK KFL interactions. Our results reveal the disordered nature of both linkers and confirm the presence of a central helix through secondary NMR chemical shifts. Furthermore, we show that both FAK KFLs can undergo phase separation under macromolecular crowding conditions. By directly comparing these linkers, our data advance the understanding of their specific functions and contribute to the emerging evidence that proteins in the focal adhesion complex can also undergo phase separation mediated by their disordered segments.

METHODS

Cloning. The cDNA sequences of Pyk2-KFL_{693–868} (residues 693–868 of Pyk2, Figure S1) and Fak1-KFL_{691–913} (residues 691–913 of Fak1, Figure S1), were cloned into the pET-15b expression vector between the *NdeI* and *XhoI* restriction sites, allowing protein production with an N-terminal 6xHis tag. The cDNAs of Pyk2-KFL_{788–830} and Fak1-

KFL_{791–834}, FAK KFL peptides, were cloned into a modified version of the pETNKI-HisSUMO3-LIC-Kan vector between the *BsaI* and *XbaI* restriction sites, allowing expression with an N-terminal SUMO tag cleavable by the SUMO protease.¹⁸ These expression vectors were transfected into *Escherichia coli* BL21 DE3 bacterial cells.

Protein Expression and Purification. The 6xHis-Pyk2-KFL_{693–868} and 6xHis-Fak1-KFL_{691–913} domains were produced in *E. coli* and then purified. The bacteria were grown at 37 °C until the absorbance at 600 nm reached 0.8, then recombinant protein production was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside. The culture was maintained at 37 °C for 3 h. Proteins requiring ¹⁵N or ¹⁵N–¹³C isotopic labeling were produced in M9 medium supplemented with ¹⁵NH₄Cl or ¹⁵NH₄Cl and ¹³C-glucose, respectively. After centrifugation of the bacterial culture at 6,000 g for 20 min to collect the bacteria, the protein extract was obtained using a homogenizer (Emulsiflex) and centrifugation at 20,000 g for 30 min to remove the insoluble fraction. The cell extract was then applied to a nickel resin for affinity chromatography (Cytiva Histrap HP 1 mL) and retained proteins were eluted with elution buffer 50 mM sodium phosphate pH 7.5, 150 mM NaCl and 500 mM imidazole. These proteins were frozen at –80 °C for later use, in the elution buffer for 6xHis-Fak1-KFL_{691–913} or after buffer exchange to 250 mM sodium phosphate (NaPi) buffer pH 6.2, 100 mM NaCl and 2 mM dithiothreitol (DTT) for 6xHis-Pyk2-KFL_{693–868}.

The 6xHis-SUMO-Pyk2-KFL_{788–830} and 6xHis-SUMO-Fak1-KFL_{791–834} were produced and purified as described above for 6xHis-FAK-KFLs. Cleavage was then performed by addition of the SENP2 protease to eliminate the 6xHis-SUMO fusion domain while the excess of imidazole was removed by overnight dialysis at 4 °C. The dialysis buffer for the FAK KFL peptides has been optimized to avoid precipitation problems, the best being a 20 mM HEPES buffer pH 7.5, 200 mM NaCl, 2 mM DTT. The cleaved peptides were then applied to a nickel affinity resin and then recovered by washing of the nonretained fraction. If necessary, proteins were concentrated using centrifugal concentrators (Vivaspin, Sartorius). Desalting columns (Zeba Spin Desalting Columns, Thermo Scientific) or dialysis membranes (SpectrumLabs) were used for buffer exchange. Proline rich peptides PRR1 and PRR2 from FAK KFLs (Figure S1) were prepared by solid phase synthesis (Genecust, France).

NMR Experiments. NMR experiments were performed at 20 °C using a Bruker 900 MHz spectrometer equipped with a triple resonance cryogenic probe. Measurements were performed with 250 μM of FAK KFLs or FAK KFL peptides, in buffer 250 mM NaPi pH 6.25, 100 mM NaCl, 0.5 mM Tris(hydroxypropyl)phosphine (THP), 10% D₂O for Pyk2-KFL_{693–868} or in buffer 20 mM NaPi pH 6.9, 50 mM NaCl, 170 mM imidazole, 10% D₂O for Fak1-KFL_{691–913} and in buffer 20 mM Hepes pH 7.4, 200 mM NaCl for the peptides, supplemented with 2 mM DTT for Pyk2-KFL_{788–830} peptide.

Two-dimensional (2D) Heteronuclear Single-Quantum Correlation (HSQC) NMR experiments were performed that required ¹⁵N isotope labeling of the analyzed proteins, except for the synthetic peptides for which the natural isotope abundance of ¹⁵N at 2.5 mM peptide concentration was used. In addition, a series of three-dimensional (3D) experiments were recorded with ¹⁵N–¹³C labeled FAK KFLs and FAK KFL peptides, including HNCA, HNCO, HNCACB, HNCACO,

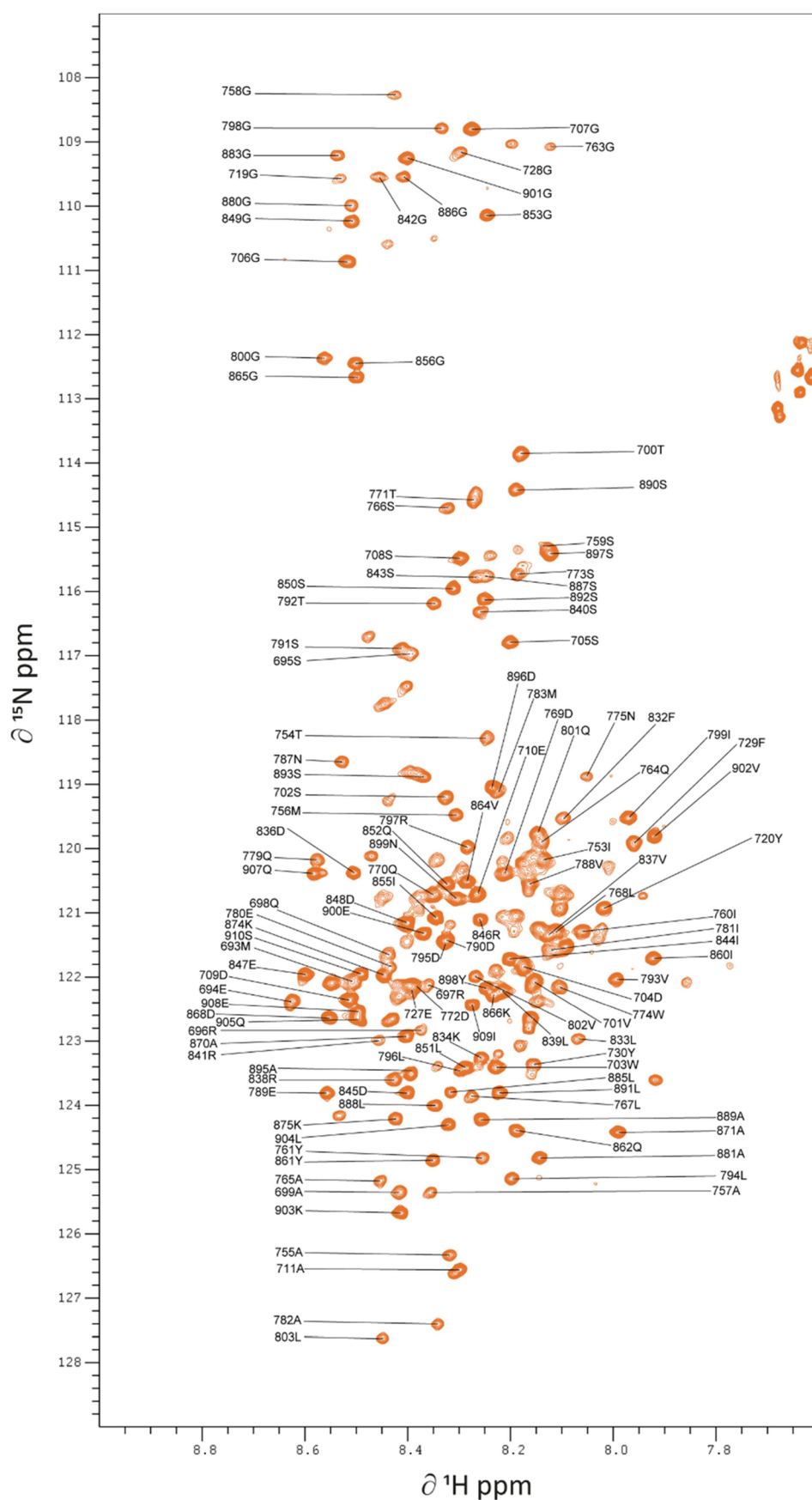


Figure 2. ^1H , ^{15}N HSQC two-dimensional full spectrum of Fak1-KFL₆₉₁₋₉₁₃. Resonance assignment is indicated.

HN(CO)CA, CBCA(CO)NH and (HACA)CO(CA)NH. The reference for the ^1H chemical shift is established relative to the methyl protons of TMSP (trimethylsilyl propionate), and the chemical shifts at ^{15}N and ^{13}C are referenced indirectly. All spectral data were recorded and processed using Topspin software (Bruker Biospin, Rheinstetten, Germany) and then analyzed using NMRFAM-SPARKY software (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco) or with CcpNmr (University of Leicester). The $\delta 2\text{D}^{19}$ Webserver (<https://www-cohsoftware.ch.cam.ac.uk/>) and the TALOS+²⁰ Webserver (<https://spin.niddk.nih.gov/bax/software/TALOS/>) were used to infer the secondary structure propensity of individual amino acids in the FAK KFLs and FAK KFL peptides as a function of protein sequence based on the assigned ^1HN , ^{15}N , $^{13}\text{C}\alpha$, $^{13}\text{C}\beta$, and $\text{H}\alpha$ chemical shifts. The $\delta 2\text{D}$ analysis provides the percentage of occupancy of a secondary structure element for each residue. TALOS+ predicts protein backbone torsion angles from NMR chemical shifts and an estimated backbone order parameter S_2 which provides a measure of mobility.²¹ In both cases, the experimental chemical shifts are compared with values from a reference database of high-resolution native state conformations for which chemical shift measurements are available. NMR assignment have been deposited in the BioMagResBank <https://bmr.io/> with BMRB entries nr 52627 for 6xHis-Fak1-KFL_{691–913}, Fak1-KFL_{791–834} and nr 52693 for 6xHis-Pyk2-KFL_{693–868}, Pyk2-KFL_{788–830}.

Size Exclusion Chromatography-Multiangle Light Scattering. Size exclusion chromatography (SEC) was performed at 20 °C on a Superdex 75 3.2/300 or increase 10/300 GL column at a flow rate of 0.1 or 0.7 mL/min, respectively, on a Shimadzu system (LabSolutions). Elution was monitored by UV, multiangle light scattering (Wyatt TREOS II, Wyatt Technology) and refractive index measurements, which were analyzed with the ASTRA 7.3 software. The composition of the elution buffer was chosen to optimize the protein solubility, in our hands: for the FAK KFL peptides, it contained 20 mM HEPES pH 7.5, 200 mM NaCl, and 2 mM DTT; for 6xHis-Pyk2-KFL_{693–868}, 250 mM NaPi pH 6.2 with 100 mM NaCl and 2 mM DTT; and for 6xHis-Fak1-KFL_{691–913}, 50 mM sodium phosphate pH 7.5 with 150 mM NaCl and 500 mM imidazole. The protein concentration for all samples was adjusted to 100 μM in the elution buffer prior 50 μL injection.

CD and Fluorescence Spectroscopies. Circular dichroism (CD) measurements were performed using a Jasco J-1500 spectropolarimeter (Jasco, U.K.) equipped with a Peltier-controlled thermo-stated cell holder at 25 °C. Samples were prepared to final concentrations of 0.90 μM Pyk2-KFL_{693–868} in 25 mM NaPi buffer pH 6.2, 10 mM NaCl and 0.2 mM DTT and 0.75 μM 6xHis-Fak1-KFL_{691–913} in 2.5 mM NaPi, 7.5 mM NaCl and 25 mM imidazole. Measurements were performed with the addition of trifluoroethanol (TFE) at concentrations ranging from 0 to 70%. Far-UV CD spectra (195–260 nm) were recorded in a 1 mm path length quartz cell (Hellma Analytics) with a minimum of 5 scans averaged per spectrum. The buffer baseline was subtracted from each spectrum. The spectra were smoothed by neighborhood averaging using four window points. Fluorescence spectroscopy measurements were performed using a Jasco FP-8200 spectrofluorometer (Jasco, U.K.) equipped with Peltier temperature control at 25 °C. Tryptophan emission spectra were obtained with an excitation wavelength of 280 nm and slits set at 5 nm for both excitation

and emission. Measurements were taken every 0.5 nm from 290 to 420 nm at medium sensitivity with a scan rate of 200 nm/min. For each spectrum, three scans were averaged. Samples of 6xHis-Pyk2-KFL_{693–868} and 6xHis-Fak1-KFL_{691–913} diluted in H_2O to concentrations ranging from 0 to 1.4 μM were used for these experiments.

Spectrophotometric Turbidity and Dynamic Light Scattering Assays. Liquid–liquid phase separation (LLPS) was investigated in samples with a concentration of 6xHis-Pyk2-KFL_{693–868} or 6xHis-Fak1-KFL_{691–913} in the range of 4 to 20 μM , in NaPi pH 7.4, 30 or 300 mM NaCl buffer, by adding PEG (polyethylene glycol) 10,000 at a concentration in the range of 0–14% (w/v). The formation of LLPS (droplets) was monitored by light absorption at 400 nm using the SPECTROStar Nano Microplate Reader (BMG Labtech, Ortenberg, Germany) and Dynamic Light Scattering (DLS) on a Nano Series Nano S (Zetasizer) instrument. Turbidity was measured at 37 °C for samples in 96-well plates with transparent bottoms and scattered light intensity at 22 °C, in Ultra-Micro Cell for Nano (MALVERN) filled with 60 μL of solution. Measurements were performed within 20 min of sample preparation. DLS data were collected as 10 acquisitions of 10 s for each point and analyzed using the ZETASIZER software.

Preparation of Fluorescently Labeled Proteins. 100 μM of recombinant 6xHis-Pyk2-KFL_{693–868} or 6xHis-Fak1-KFL_{691–913} were incubated with a 3 molar excess of Atto565 NHS ester dye (SIGMA-Aldrich) in 50 mM NaPi pH 7.4, 30 mM NaCl buffer. The labeling mixture was incubated for 2 h at 4 °C in the dark before the reaction was quenched by addition of 10 mM Tris–HCl pH 7.4. 100 μM of recombinant 6xHis-Pyk2-KFL_{693–868} was incubated with 300 μM of 5(6)-carboxytetramethylrhodamine C6 (TAMRA) maleimide (Euromedex) for 2 h at room temperature. The reaction was quenched with 2 mM DTT after a 2-h incubation period at 20 °C. Excess of dyes was removed by buffer exchange using a Zeba Spin Desalting Columns (Thermo Scientific).

Brightfield and Fluorescence Microscopy. The FAK KFL droplets were prepared as described here above and imaged immediately after the addition of 12% PEG. Briefly, 6 μL of the solution was applied to a glass-bottomed microscope slide ($\mu\text{-Slide}$, Ibidi), precoated with poly-D-lysine (0.5 mg/mL, Sigma) and laminin (10 $\mu\text{g/mL}$, Sigma). Imaging was performed on a wide field microscope (Leica AF600) using a 100 $\times$ oil objective with bright field or fluorescence microscopy (using a FITC filter) and on a confocal microscope using a Zeiss AxioObserver Z1 (Yokogawa CSU-X1 spinning disk, sCMOS Photometrics Prime 95B camera).

RESULTS

FAK KFLs Are Mainly Disordered. We have produced and purified FAK KFLs (Pyk2-KFL_{693–868} and Fak1-KFL_{691–913}, Figures 1 and S1) as fusions to an N-terminal 6 His-Tag and next assigned the chemical shift values of their amino acid resonances using Nuclear Magnetic Resonance spectroscopy (NMR) to obtain structural information at the residue level. The 2D ^1H – ^{15}N HSQC NMR spectra of FAK KFLs showed a narrow resonance dispersion on the proton scale consistent with a disordered state (Figures 2, S2, and S3). The assignment proved difficult because of the size of the FAK KFLs, signal overlap due to the narrow dispersion and the presence of many broad resonances that gave low signal intensity. The high percentage of proline residues (Figure S1)

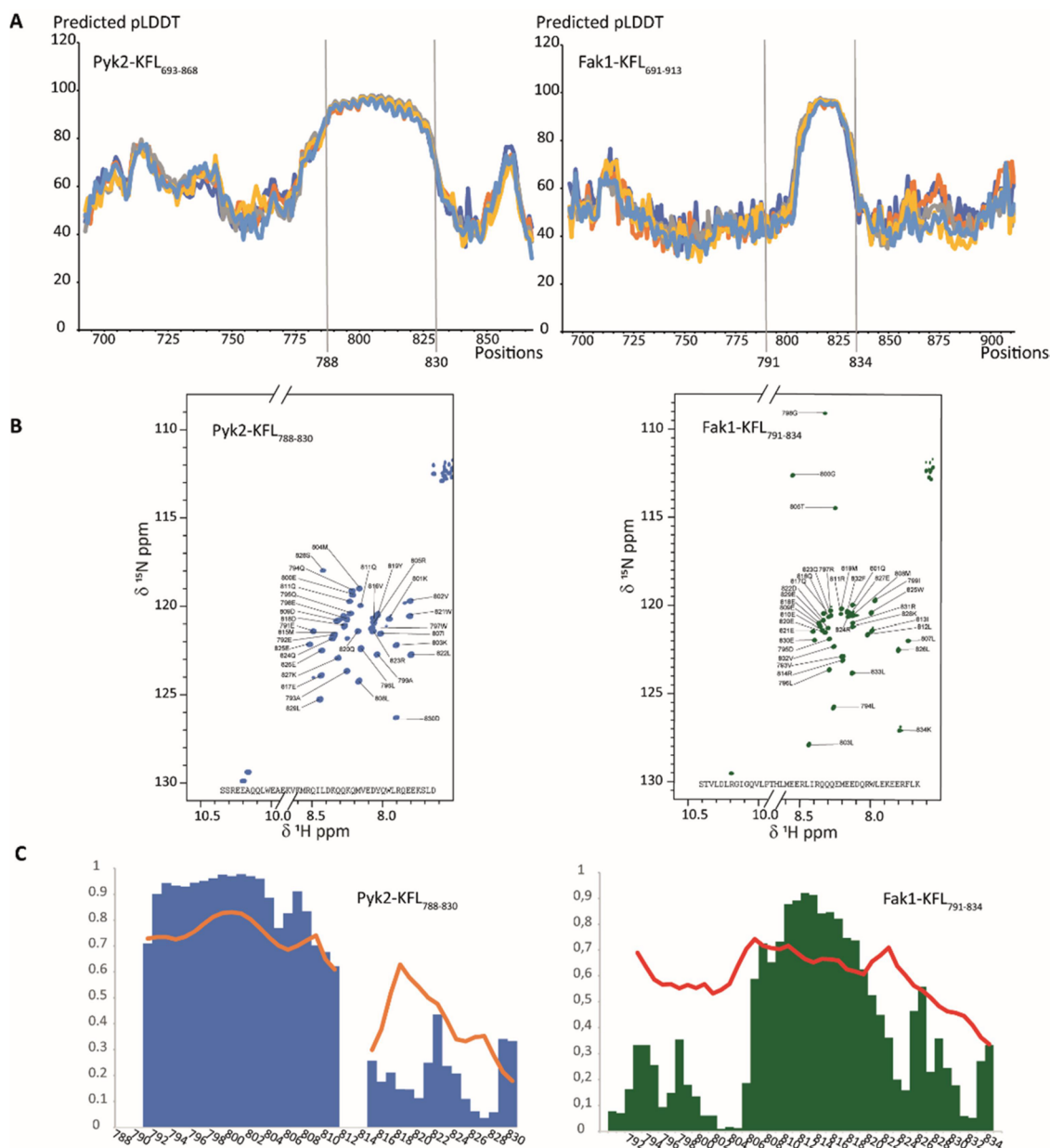


Figure 3. Secondary structure analysis (A) AlphaFold molecular prediction of the conformation of the FAK KFLs: the pLDDT (predicted local-distance difference test) score value per position, for five models, shows high accuracy of the helical prediction of Pyk2-KFL_{788–830} and Fak1-KFL_{791–834} while the rest of the domain is disordered. (B) 2D ¹H, ¹⁵N HSQC of Pyk2-KFL_{788–830} and Fak1-KFL_{791–834} peptides labeled with the resonance assignment. The ¹H scale was shortened for visualization of the tryptophan side-chain signals beyond 9.5 ppm. (C) NMR secondary chemical shift analysis (of ¹H_α, ¹³C_α, ¹³C_β, ¹³CO, ¹⁵N, and ¹H_N) by TALOS revealed helical content. Positive values are indicative of a preferential helical conformation (blue or green bars) while the index value is indicative of the preference to adopt this conformation. An estimated backbone order parameter S₂ derived from the chemical shifts is shown as orange or red lines. Disordered regions have a low S₂.

also increased the assignment challenge, as these amino acid residues are not detected in ¹H–¹⁵N experiments. Thus, 2 short peptides corresponding to proline-rich regions were initially assigned to serve as anchoring points for the sequential assignment of ¹H_N, ¹⁵N, ¹H_α, ¹³C_α, ¹³CO, and ¹³C_β NMR signals of the FAK KFLs (Table S1). Examination of the FAK KFL assignment coverage showed that the missing resonances, due to broadening beyond detection, corresponded mainly to a protein segment encompassing residues 765–828 of Pyk2-KFL_{693–868} and 805–830 of Fak1-KFL_{691–913} (Figure S4A,B).

Available assignments for both KFLs nevertheless allowed to calculate secondary structure propensity based on the chemical

shift values, which showed that no secondary structure elements could be detected in the regions with assigned signals because all the chemical shift values resembled mostly random coil peptide resonances (Figure S4A,B).

In agreement, both FAK KFLs were mostly predicted to be disordered, using PONDR (Figure S4C) and AlphaFold (Figures 1 and 3A).

This was further confirmed by circular dichroism secondary structure analysis of Pyk2-KFL_{693–868} and Fak1-KFL_{691–913} that showed only 23 and 10% of helical content respectively, in agreement with the AlphaFold predictions (Figure S5, green

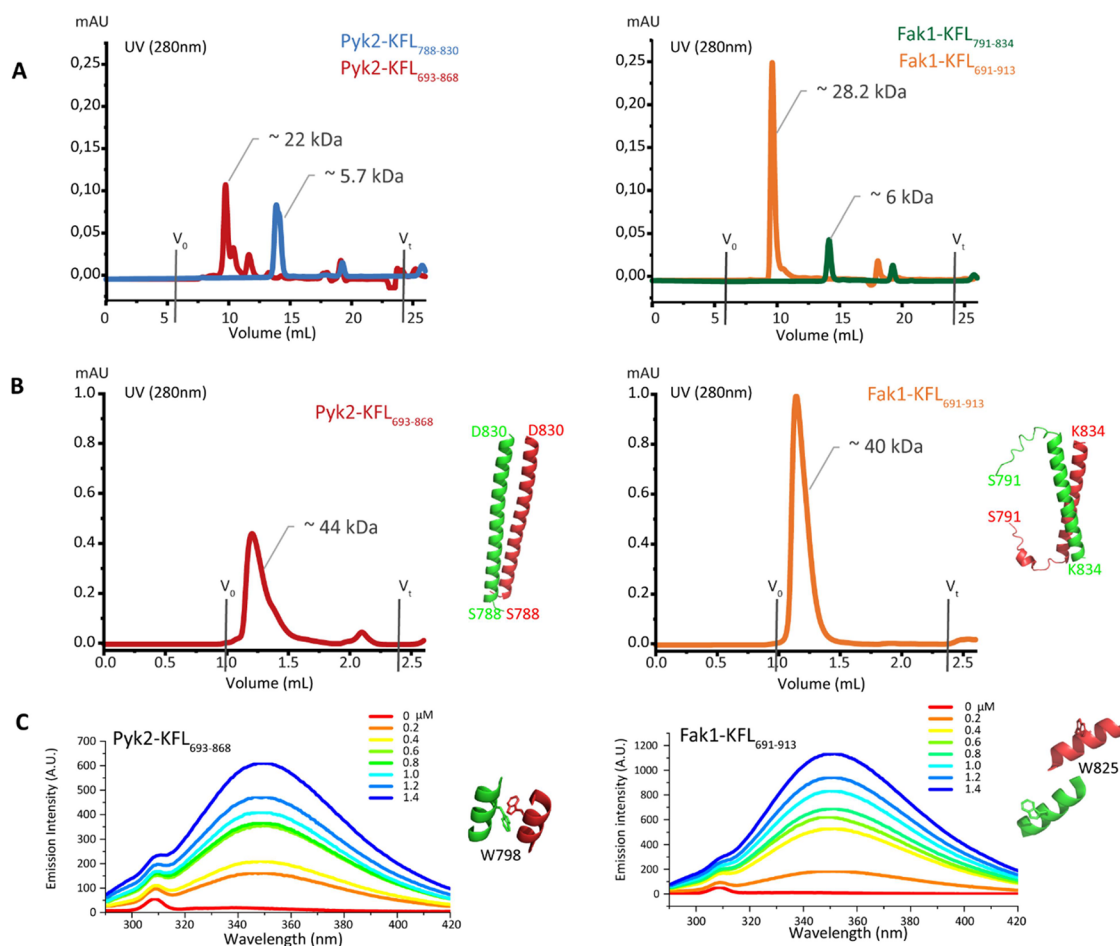


Figure 4. Monomer–dimer forms of FAK KFL and KFL peptides (A, B) SEC profiles ((A) S75 resin, 24 mL bed volume and (B) S75 resin, 2.4 mL bed volume) of Pyk2-KFL_{693–868} in 250 mM NaPi, 100 mM NaCl, pH 6.2, of Fak1-KFL_{691–913} in 50 mM NaPi pH 7.4, 100 mM NaCl, 500 mM imidazole and of Pyk2-KFL_{788–830} and Fak1-KFL_{791–834} peptides in buffer 20 mM HEPES pH 7.5, 200 mM NaCl, and 2 mM DTT. Molecular weights determined by MALS measurements are given. (C) Fluorescence emission after excitation at 280 nm of Pyk2-KFL_{693–868} and Fak1-KFL_{691–913}. (B, C) Cartoon representation of the detail of a representative AlphaFold predicted structure of the Pyk2-KFL_{693–868} and Fak1-KFL_{691–913} dimer is shown (see Figure S6). In (C) the tryptophan side chains are shown as sticks.

curves, 0% TFE). In silico and experimental analysis thus concurred to define the FAK KFLs as mainly disordered.

FAK KFLs Contain a Segment with a Helical Conformation. We next turned our attention to the unassigned regions, using a strategy of peptide coverage to obtain further information on chemical shift values and secondary structure. Peptides were thus chosen corresponding to the regions of FAK KFLs that remain unassigned (Figure S4A,B), additionally informed by the AlphaFold analysis that predicted formation of a helical conformation within these regions with good confidence (Figure 3A). The resulting peptides had 43 and 44 amino-acid residue sequence length, namely Pyk2-KFL_{788–830} and Fak1-KFL_{791–834}, respectively. These KFL peptides were produced with double-¹⁵N–¹³C labeling using recombinant expression with a N-terminal SUMO domain fusion, next removed by enzymatic cleavage using the SUMO protease. Both peptides gave spectrum quality compatible with assignment (Figure 3B). Analysis of the secondary chemical shifts based on this assignment confirmed the tendency to form helical conformations in these peptides (Figure 3C). Consistent with this, titration with trifluoroethanol (TFE), a compound known to stabilize transient helices, resulted in a dose-dependent increase in the negative CD signal at 208 and 222 nm, characteristic of α -helix

formation, in the FAK KFL solutions (Figure S5). Comparison of the resonances of the corresponding residues in the FAK KFL peptides and the complete FAK KFLs confirmed that most of the resonances of the peptides were missing in the spectra of the FAK KFLs (Figures S2 and S3).

FAK KFLs Form Multimeric Assemblies. While the resonance broadening observed in the spectra of the FAK KFLs can be partially attributed to transient helix dynamics, dimerization, as reported,¹¹ could also contribute to this broadening. To explore this possibility, we next investigated the quaternary state of the KFLs and the KFL peptides by SEC-MALS (size exclusion chromatography-multiangle light scattering) (Figure 4A,B). The absorption peak at 280 nm of the FAK KFLs and the KFL peptides separated on a 24 mL column corresponded to polypeptides with masses indicative of monomeric forms as estimated by MALS. The Pyk2-KFL_{788–830} and Fak1-KFL_{791–834} peptides indeed eluted as a single peak (Figure 4A) corresponding to peptides with molecular weights of 5747 ± 126 Da and 5996 ± 996 Da, respectively, consistent with a monomeric state (the calculated MW of Pyk2-KFL_{788–830} is 5481 Da and that of Fak1-KFL_{791–834} is 5523 Da). Similarly, Pyk2-KFL_{693–868} and Fak1-KFL_{691–913} eluted with estimated molecular weights of $22,000 \pm 108$ Da and $28,200 \pm 109$ Da, respectively (the calculated

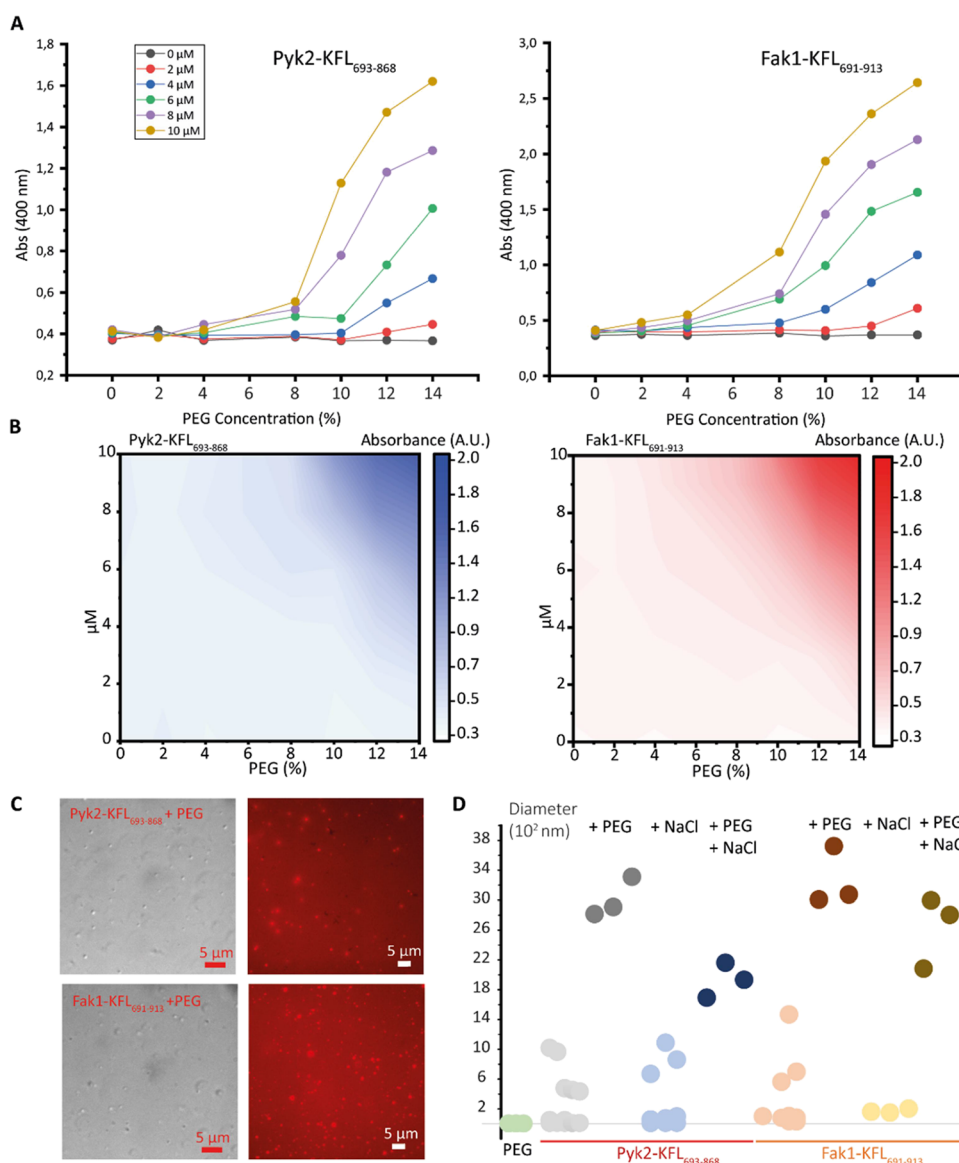


Figure 5. Characterization of in vitro FAK KFL LLPS formation in the presence of PEG (polyethylene glycol). (A) Plot of PEG-induced LLPS followed by turbidity (absorbance at 400 nm) at increasing concentrations -0 μ M (blue curve), 5 μ M (orange), 10 μ M (green), 15 μ M (yellow), 20 μ M (light blue) of Pyk2-KFL₆₉₃₋₈₆₈ and Fak1-KFL₆₉₁₋₉₁₃ and at increasing concentrations of PEG to reach 14%. Averaged values are shown, corresponding to three replicates of FAK KFL droplet preparation for each of the conditions tested. Turbidity measurements were performed within 20 min after mixing at 37 °C using buffer 50 mM NaPi, pH 7.4, 30 mM NaCl. (B) Phase diagrams of FAK KFLs as in (A). Color coding represents average absorbance values at 400 nm. (C) Representative bright-field microscopy images of 4 μ M Pyk2-KFL₆₉₃₋₈₆₈ or Fak1-KFL₆₉₁₋₉₁₃ in the presence of 12% PEG in buffer 50 mM NaPi pH 7.4, with 30 mM NaCl and fluorescence microscopy images in the same buffer conditions (for Atto565-labeled FAK KFLs). Scale bar: 5 μ m. (D) Characterization of the hydrodynamic diameter of FAK KFL condensates by dynamic light scattering measurements. Representative size distribution (diameter) of PEG alone and Pyk2-KFL₆₉₃₋₈₆₈ or Fak1-KFL₆₉₁₋₉₁₃, without and with 12% PEG (+PEG) in buffer 50 mM NaPi pH 7.4, with 30 mM NaCl or 300 mM NaCl (+NaCl). Measurements for one data point are plotted on a vertical axis and at least 3 replicates are shown for each experimental condition.

MW of 6xHis-Pyk2-KFL₆₉₃₋₈₆₈ is 22 847 Da and that of 6xHis-Fak1-KFL₆₉₁₋₉₁₃ is 26 561 Da). However, when a smaller 2.4 mL column was used, with less dilution of the injected sample, Pyk2-KFL₆₉₃₋₈₆₈ and Fak1-KFL₆₉₁₋₉₁₃ eluted with estimated molecular weights of 44.2 ± 5.7 and 40 ± 1 kDa, respectively (Figure 4B), which are consistent with the expected values for dimeric FAK KFLs. AlphaFold predictions of a dimeric FAK KFL model suggested that the helical peptides may serve as the primary contact points between subunits, forming a coiled-coil interface (Figures 4B and S6). However, tryptophan fluorescence of Pyk2-KFL₆₉₃₋₈₆₈ (2 Trp, one in the Pyk2-KFL₇₈₈₋₈₃₀ peptide) and Fak1-KFL₆₉₁₋₉₁₃ (4 Trp, one in the

Fak1-KFL₇₉₁₋₈₃₄ peptide) evidenced emission bands with maxima at ~ 354 and 357 nm (Figure 4C), respectively, consistent with exposure of these residues to a polar environment, indicating the dynamic nature of the dimer.

FAK KFLs Undergo Phase Separation under Macromolecular Crowding Conditions. Many proteins with intrinsically disordered conformations can undergo liquid-liquid phase separation or demixing from solution to form condensates under crowding conditions.^{12,13} Therefore, we investigated PEG-induced LLPS formation by monitoring the light absorbance (turbidity) at 400 nm of FAK KFL samples at different concentrations of PEG used as a crowding agent

(Figure 5). We observed that the increase in turbidity was dependent on the increase in protein and PEG concentration (Figure 5A,B). Indeed, imaging revealed the formation of numerous spherical and micron-sized droplets, under PEG-promoted crowding conditions, whereas no particles were observed in the absence of crowding (Figures 5C and S7A,B). In addition, DLS experiments confirmed that the addition of PEG resulted in an increase in the hydrodynamic diameter of both FAK KFLs, compatible with droplet formation (Figure 5D). Droplet formation was reduced by the addition of high salt concentrations at up to 300 mM NaCl, as indicated by the decrease in the number of droplets observed by imaging (Figure S7C) and the decrease in the average diameter of the droplets measured by DLS (Figure 5C).

DISCUSSION

Our results show that while both FAK KFLs have similar properties as long disordered domains, they also exhibit distinct features, particularly with respect to the lower tendency for helical segment formation in the central region of Fak1-KFL_{691–913}. Notably, the AlphaFold models successfully capture this difference, which is further supported by secondary chemical shift analysis of the NMR signals and CD spectroscopy. Since the Pyk2 helical peptide (Pyk2-KFL_{788–830}) has been shown to interact with calmodulin¹¹ to mediate its specific calcium-sensing function, this structural difference may reflect the functional specificity of Pyk2.

Here, we observed dimer formation in both FAK KFLs by SEC-MALS analysis, a process likely to be mediated by coiled-coil interactions of the helices located in the KFL peptides, as suggested by AlphaFold modeling (Figure S6). However, the confidence of the models is low outside of the helical KFL peptides, and additional interactions may occur between other segments that are not captured by these models. Indeed, other segments of the FAK KFLs showed poor coverage of the chemical shift assignment, due to broadening of the NMR signals, which, similar to the segment corresponding to the KFL peptides, could indicate the formation of transient helices and/or intermolecular contacts (e.g., Fak1-KFL_{730–740}, Figure S4B,C). Consistently, it has been proposed that the Pyk2 KFL_{728–839} fragment forms a fuzzy and dynamic self-association, involving residues distributed along the sequence.¹¹ In addition, the dispersion of particle sizes observed by DLS was large (Figure 5D), which may indicate the potential to form higher oligomeric forms.

Here, we have observed that both FAK KFLs can independently undergo LLPS when subjected to PEG-induced crowding conditions. However, it remains to be further investigated how this might be relevant in the context of the full-length multidomain protein and of multiple partners in the cell. The full-length Fak1 protein has previously been described to drive LLPS, at concentrations as low as 40 nM.¹⁴ The KFL region of Fak1 did not appear to drive the condensate formation under these noncrowding conditions, but rather the dimerization of the FERM domain.¹⁴ Interaction with paxillin, another component of the adhesion complex, was shown to enhance the condensate formation by Fak1, whereas kindlin and talin-head did not. The complex web of intra- and intermolecular interactions of Fak1 thus appears to regulate the LLPS in a complex manner that is not yet fully understood. Increasing the salt concentration did not completely disrupt droplet formation by either FAK KFL, although a decrease in their average size is observed by DLS and fewer droplets are

imaged (Figures 5D and S7C), suggesting that the weak protein–protein interactions driving LLPS are electrostatic, but not only in this context.²² Taken together, these results are consistent with previous observations that coiled-coil interactions between proteins promote LLPS by facilitating multimerization.^{23–25}

CONCLUSIONS

These data will be of interest in investigating interactions that the FAK KFLs may have with other protein partners, such as proline-rich region binding proteins. They add to the growing evidence for the involvement of phase separation in focal adhesion formation. Overall, this work demonstrates the versatility of the FAK KFL regions of both Pyk2 and Fak1, whose role is not limited to the mere structural aspect of connecting two globular domains, but is also involved in the regulation of protein functions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.biochem.4c00677>.

Protein sequence alignment; two-dimensional NMR spectra of Pyk2-KFL; two-dimensional NMR spectra of Fak1-KFL; peptide chemical shift assignments; secondary structure propensity; circular dichroism spectra; AlphaFold prediction of dimer formation; and condensate characterization (PDF)

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Notes

The authors declare no competing financial interest.

Focal Adhesion Kinase 1 (Fak1)_Homo sapiens 1052 amino acid residues - UniProtKB: locus FAK1_HUMAN, accession Q05397. Focal Adhesion Kinase 2 (Pyk2)_Homo sapiens 1009 amino acid residues - UniProtKB: locus FAK2_HUMAN, accession Q14289

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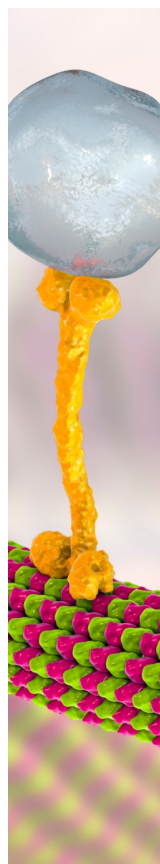
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