Structural and functional analysis of target recognition by the lymphocyte adaptor protein LNK

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The SH2B family of adaptor proteins, SH2-B, APS, and LNK are key modulators of cellular signalling pathways. Whilst SH2-B and APS have been partially structurally and biochemically characterised, to date there has been no such characterisation of LNK. Here we present two crystal structures of the LNK substrate recognition domain, the SH2 domain, bound to phosphorylated motifs from JAK2 and EPOR, and biochemically define the basis for target recognition. The LNK SH2 domain adopts a canonical SH2 domain fold with an additional N-terminal helix. Targeted analysis of binding to phosphosites in signalling pathways indicated that specificity is conferred by amino acids one- and three-residues downstream of the phosphotyrosine. Several mutations in LNK showed impaired target binding in vitro and a reduced ability to inhibit signalling, allowing an understanding of the molecular basis of LNK dysfunction in variants identified in patients with myeloproliferative disease.
Hemoglobin (TPO) and erythropoietin (EPO) are hematopoietic cytokines that bind specific receptors on the surface of target cells and induce activation of the Janus kinase (JAK)-signal transducer and activation of transcription (STAT) pathway. TPO is an essential modulator of megakaryopoiesis, platelet production and stem cell quiescence, whereas EPO is required for the regulation of erythropoiesis. The Lymphocyte adaptor protein (LNK) (also SH2B3) is a member of the SH2 domain containing adaptor family of proteins, which also comprises APS (SH2B2) and SH2B (SH2B1)-5 and negatively regulates both EPO and TPO signalling via its interaction with JAK2. All three proteins contain a dimerisation domain, a pleckstrin homology (PH) domain and a Src homology 2 (SH2) domain and are involved in the regulation of various signalling pathways downstream of cytokines and growth factors.2,3,5–10

LNK is highly expressed in hematopoietic stem cells (HSCs), and deletion of LNK from HSCs leads to an increase in cell number and proliferative capacity,6 suggesting a role for LNK in regulating HSC self-renewal. Comparably, overexpression of LNK in hematopoietic progenitor cell lines restrains TPO-induced cellular proliferation, and overexpression of LNK in primary hematopoietic cells inhibits megakaryopoiesis.4,5 These findings are recapitulated in vivo with LNK-deficient mice displaying increased numbers of megakaryocytes that have enhanced TPO sensitivity as well as enhanced numbers of platelets, lymphocytes and erythroid cells.5,6 Similarly, erythroid colony-forming progenitors from the spleens of LNK deficient mice displayed increased sensitivity to EPO stimulation.5

Given the ability of LNK to negatively regulate EPO and TPO signalling, it is unsurprising that LNK loss-of-function mutations have been identified as drivers of human myeloproliferative disease.11 In addition, there is an increased incidence of LNK mutations in leukemic transformation of myeloproliferative neoplasms, suggesting LNK function may effect the severity of disease.12–14. The PH and SH2 domains of LNK are both hotspots for mutations, and are essential for LNK function. Currently the mechanism by which LNK negatively regulates signalling is poorly understood, and so the role of LNK substitutions in the onset and progression of disease is uncertain. Understanding how the LNK SH2 domain interacts with substrates may shed light on how negative regulation of various signalling molecules occurs, and why mutations in the LNK SH2 domain contribute to disease burden in patients.

LNK has also been suggested to bind a suite of other signalling proteins involved in haematopoiesis, proliferation and differentiation, including c-KIT,6 FLT3,7 c-FMS,8 and PDGFR9.

The need to establish which signalling proteins are regulated by LNK is underscored by the identification of LNK mutations in patients with a range of inflammatory, immune and haematopoietic diseases including cancer.20–25 Characterisation of the substrate recognition domain of LNK, the SH2 domain, would therefore be a step towards a comprehensive overview of the proteins and pathways that LNK negatively regulates. While the structural details of APS and SH2-B have been elegantly characterised by the Hubbard laboratory,26,27 to date there have been no structural or biochemical studies of LNK, with only a single published example of the successful expression of any part of the LNK protein and no example of the yields and purity required for an in-depth structural and biochemical analysis.

Here we present the structure of the substrate recognition domain of LNK, the SH2 domain, in complex with phosphopeptide motifs from JAK2 (pY813) and EPOR (pY454) and biochemically characterise these interactions. The LNK SH2 domain, unlike APS but similarly to SH2-B, adopts a monomeric structure and binds its target sequences in a canonical linear, extended conformation. We tested the ability of the LNK SH2 domain to bind to a number of different phosphorylated peptides from proteins proposed to be regulated by LNK, revealing the highest affinities were for sites on JAK2, JAK3 and EPOR. Studies of SH2 domain point mutations identified in patients with myeloproliferative neoplasms revealed that the SH2 domain displayed a decrease in affinity for ligands and the same mutations, when incorporated into the full-length protein had a reduced capacity to regulate signalling in vitro. Together these findings detail the specificity of the LNK SH2 domain and aid our understanding of how single point mutations affects LNK function, contributing to haematological diseases.

Results
Crystal structure of the LNK SH2 domain in complex with the JAK2 pY813 motif. We attempted a number of different bacterial and baculovirus expression systems to produce active SH2 domain from both human and mouse LNK including, Whilst GST and His6-based fusion systems were unsuccessful, a NusA fusion of the M. musculus LNK SH2 domain (using the domain boundaries from Machida et al.) yielded folded, monomeric protein once the fusion tag was removed as determined by size exclusion chromatography and thermal shift assay. The LNK SH2 domain was then co-crystallised with a 12-mer phosphopeptide corresponding to JAK2 pY813 and flanking residues (designated below as +1,+2.... and −1,−2.... relative to pTyr). Importantly, the murine LNK SH2 domain shares 92.9% identity with the H. sapiens orthologue (Supplementary Fig. 1), having high conservation across all phosphopeptide-binding residues, with only a threonine to serine substitution from human to mouse within the phosphotyrosine binding pocket. The crystal structure of this complex was solved to 1.9 Å resolution (Supplementary Table 1), revealing that the LNK SH2 domain adopts a typical SH2 domain fold comprising three central β-strands flanked by two α-helices. In addition, there is a short helix similar to that found in the SOCS and STAT proteins located just outside the SH2 domain boundary on the N-terminal side (Fig. 1a and 1b). This helix extends behind the central β sheet and is positioned over a hydrophobic patch. A leucine in this helix (Leu330) begins an LsXYP motif conserved across all three SH2B family members and inserts its sidechain into a hydrophobic pocket on this surface (Fig. 1b and Supplementary Fig. 2). Thus, this N-terminal helix may be a conserved feature across the SH2B family. Additionally, this helix appears to be conserved across several vertebrate species (Supplementary Fig. 2), suggesting this N-terminal helix may be an evolutionarily conserved feature in LNK.

Another unexpected feature is a disulfide bond formed between Cys 421 and Cys 425 of the BG loop, a feature not present in SH2B or APS; whether this would be present in vivo is unclear.

The pTyr of the JAK2 pY813 phosphopeptide inserts into the canonical phosphotyrosine binding pocket of the LNK SH2 domain, formed by Arg 343, Arg 364, Ser 366, Arg 369, His 385 and Arg 387. The phosphate moiety of pY813 forms hydrogen bonds with the invariant Arg 364 and the highly conserved Arg 343 along with the backbone amide nitrogen of Glu 367, and the sidechain of Ser 368 (Fig. 1c, d). Glu 814 of the JAK2 peptide (P +1) appears to be a key determinant of binding as it forms a hydrogen bond with Lys 384 of LNK. In addition, Leu 816 (P +2) appears to be a key determinant of binding as it forms a hydrogen bond with Lys 384 of LNK. In addition, Leu 816 (P +3) inserts into a hydrophobic pocket common in SH2 domains that is formed by Leu 386, Val 398, Leu 401, Phe 403, Phe 413, Ile 418 and Leu 420 of LNK (Fig. 1c). Numerous backbone interactions form the remainder of intermolecular contacts (Fig. 1b and Supplementary Fig. 3).
Binding of the LNK SH2 domain to phosphotyrosine motifs resembles the binding mode of SH2B and is distinct from that of APS. The SH2 domains of APS and LNK share 64.6% sequence identity (Fig. 2a), with high conservation around the phosphotyrosine binding pocket (Supplementary Fig. 4a) however the structures are notably different. APS forms a dimer (PDB ID: 1RQQ) whereas LNK is monomeric. APS dimerisation occurs via its αB helix, which is longer and extends behind the SH2 domain (Fig. 2b). The +3 hydrophobic pocket forms part of this dimer interface and as such peptide binding is considerably altered (Supplementary Fig. 4a) and differs from most SH2 domains by not adopting a linear, extended conformation.

In contrast, LNK is structurally more similar SH2B, which also binds JAK2 pY813. The SH2 domains of LNK and SH2B share 68.7% sequence identity (Fig. 2a) and the crystal structures of their SH2 domains (SH2B PDB ID: 2HDX) align with a root-mean-square deviation (RMSD) of 1.26 Å over 106 atoms (Fig. 2b). There are subtle differences in pTyr binding between these two family members despite a high degree of conservation across the peptide binding residues (Supplementary Fig. 4b). In both, the phosphate group of pY813 occupies the canonical phosphotyrosine binding pocket and is coordinated by an invariant arginine (Arg 364 in LNK), however in LNK, Arg 369 and 387 are involved in a hydrogen bonding network with Glu 372, and do not interact with the phosphate moiety. In contrast, in SH2B, the corresponding three residues form different interactions and the Arg 369 equivalent (Arg 560) forms a salt bridge with pTyr. LNK, like SH2B also displays...
Specificity for a Glu at the +1 position, however in LNK this is facilitated by a salt bridge to Lys 384, whereas in SH2B the equivalent lysine hydrogen bonds to Ser 613 which likewise hydrogen bonds to the +1 Glu.

Specificity for the LNK SH2 domain is conferred by amino acids one-residue, three-residues and five-residues downstream of the target phosphotyrosine. SH2 domains typically bind ligands with a specific preference for amino acids at the −2 to +5 positions, relative to phosphotyrosine. The LNK SH2/JAK2 pY813 structure suggests that +1 and +3 residues contribute to high affinity binding. To determine the contribution of individual residues, we made a series of mutations within the JAK2 phosphopeptide, and measured their affinity for the LNK SH2 domain via a surface plasmon resonance (SPR) competition assay (Fig. 3). The LNK SH2 domain was incubated with increasing concentrations of mutant JAK2 pY813 peptides before being passed over a chip coated with an immobilised low affinity phosphopeptide derived from a site on the intracellular domain of the IL-6 receptor, IL6ST. While the IC50 of the WT pY813 peptide was 60 nM, mutation of residues at the +1, +3, and to a lesser extent +5, positions decreased the affinity of the interaction (Fig. 3). In particular, mutation of the +1 Glu to Arg (+1R) and the +3 Leu to Phe (+3F) resulted in a >80-fold and ~25-fold decrease in affinity respectively (Table 1). The +2 residue was not mutated as the structure indicates its sidechain points towards solvent. These findings, in combination with the SH2 domain structure, indicate three residues as key binding determinants; Glu 814, Leu 816 and Glu 818, in addition to pTyr 813.

Identification of phosphotyrosine binding motifs in JAK3, EPOR, FLT3 and c-KIT. The LNK SH2 domain has been proposed to interact with a suite of phosphorylated sites on various signalling proteins. Here, using an SPR competition assay,
we examined binding of the LNK SH2 domain to those suggested sites from c-KIT, c-FMS, FLT3 and PDGFR and to all intracellular sites from EPOR and TPOR. In addition, binding to the activation loops of JAK2 and IRK, and pT785 of JAK3 were also examined. Although biophysical methods do not prove that an interaction exists in vivo, they provide a powerful tool to show which interactions do not occur. Given that SH2 domains will bind to most phosphorylated sequences to some degree, in line with the literature on SH2 domains and their targets, we applied a threshold cut-off of 2000 nM, where an IC50 over this value indicates a physiologically irrelevant interaction. As shown in Table 2, we identified two high-affinity phosphorylase motifs from JAK3 and EPOR in addition to several moderate-affinity motifs from EPOR, FLT3 and c-KIT. These sequences all contain a hydrophobic residue in the +1 position. Peptides with an IC50 greater than 2000 nM generally either lacked a hydrophobic residue at this position or contained an Arg at the +1 which we had previously shown abolished binding (Fig. 3). Despite previous reports implicating pTyr residues of PDGFR and c-FMS, as LNK ligands, in our hands, we did not observe binding of the LNK SH2 domain to phosphopeptides corresponding to these residues.

Crystal Structure of the LNK SH2 domain in complex with the EPOR pY454 motif. To understand how the EPOR pY454 peptide, which contains a leucine at the +1 position, could bind the LNK SH2 domain with high affinity, we determined the complex structure to 2.35 Å resolution (Supplementary Table 1). Overall, the structure is very similar to the LNK SH2/JAK2 pY813 structure with an RMSD of 1.0 Å over 111 residues (DALI) (Fig. 4a and Supplementary Fig. 5). The LNK SH2 domain accommodates the leucine of the EPOR pY454 peptide at the key +1 position in a subtly different manner to the glutamate of the JAK2 pY813 peptide. The aliphatic portions of both the +1 glutamate and leucine sit on a hydrophobic surface of LNK, however the glutamate sidechain bends so that the carboxylate faces away and forms a salt bridge with Lys 384 (Fig. 4b, c). The side chain of the EPOR pY454 +1 leucine is accommodated by a shift in the BG loop of LNK by ~5 Å, although the exact positioning may be influenced by an adjacent crystal contact. In concordance with their similar binding modes, the surface area buried by each peptide is similar (~580 Å).

Mutations found in human LNK impair target binding. Several substitutions in LNK have been linked with diseases including haematological cancers, autoimmune disorders, and heart disease. Most of these occur within the PH domain however substitutions located within the SH2 domain have also been

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**Table 1 Peptide sequences and IC50 values from mutant JAK2 pY813 competition assay.**

| Mutant | Residues | IC50 (nM) ± SEM |
|--------|----------|----------------|
| WT     | pY       | 60 ± 20        |
| −1A    | pY       | 150 ± 20       |
| +1A    | pY       | 430 ± 90       |
| +1R    | pY       | >5000          |
| +3F    | pY       | 1430 ± 300     |
| +4A    | pY       | 90 ± 25        |
| +5A    | pY       | 300 ± 90       |

Target phosphorylase and mutated residues are highlighted in bold. Data are shown as mean ± SEM of technical replicates from at least two independent experiments (WT, five samples from n = 3 independent experiments, all mutants, four samples from n = 2 independent experiments).

**Table 2 Peptide sequences and IC50 values from phosphopeptide competition assay.**

| Protein | Phosphotyrosine | Sequence | IC50 (nM) ± SEM |
|---------|-----------------|----------|----------------|
| JAK2    | pY1007/pY1008  | A A      | >10,000        |
| JAK3    | pY1007/pY1008  | A -      | >10,000        |
| IRK     | pY552          | E E      | >10,000        |
| MPL     | pY626          | A N      | >10,000        |
| EPOR    | pY485          | S D      | >10,000        |
| FLT3    | pY572          | Y K      | >10,000        |

Target phosphotyrosine and mutated residues are highlighted in bold. Data are shown as mean ± SEM of technical replicates from at least two independent experiments. Target phosphotyrosine and mutated residues are highlighted in bold.
investigated whether the incorporation of these mutations (Supplementary Fig. 6). V402M, R415C and R415H (V374M R387C/H in mouse respectively) around the phosphotyrosine binding site in the LNK SH2 domain, is positioned in a hydrophobic surface on the LNK SH2 domain (left). The +1 Glu of the JAK2 pY813 peptide is positioned over the same surface, however the carboxylate also forms a salt bridge interaction with Lys 384 of LNK (middle). The LNK SH2 BG loop of the EPOR pY454 bound structure is displaced by approximately 5 Å in comparison to the JAK2 pY813 bound structure (red) (right).

Fig. 4 Comparison of the LNK SH2 domain bound to JAK2 pY813 and EPOR pY454 phosphopeptides. a Alignment of the backbone of the WT LNK SH2 domain from the JAK2 pY813 bound structure (red) and the EPOR pY454 bound structure (purple) with secondary structural features indicated. b The pY813 JAK2 (black) and EPOR pY454 (white) peptides bound to the SH2 domain show a high degree of similarity. c The +1 Leu of the EPOR pY454 peptide is displaced by approximately 5 Å in comparison to the JAK2 pY813 bound structure (red) (right).
via JAK2, although it is possible that other domains in the protein may modulate an interaction with this receptor. To date, we have been unable to purify the full-length protein.

The documentation of LNK mutations in patients with MPNs has generated interest in understanding the role of LNK in haematological diseases. Although mutations in LNK are not commonly found as drivers of MPNs, evidence has emerged that there is an overrepresentation of LNK mutations in blast-phase MPN, suggesting a role in leukemic transformation13,14. Here we investigated three single amino acid substitutions located within the LNK SH2 domain that had been identified in MPNs or the closely related disease, idiopathic erythrocytosis; V402M, R415C, and R415H (V374 and R397 in mouse)11,15,39,40,42. Of these, V374M had the most severe effect on peptide binding. V374 is positioned directly underneath the pTyr ring, and pTyr binding is presumably blocked by the larger methionine side chain. The effect of the R387C/H LNK SH2 domain variants were more subtly. We observed a slight loss in thermal stability and affinity with these two mutants but they were still able to bind phosphorylated motifs to some extent. We hypothesise that the loss of affinity for JAK2 pY813. Data are displayed as mean ± SD from n = 3 independent experiments where data were pooled from three experiments with each data point representing the mean of triplicate replicates of a single transfection normalised to the mean RLU (relative light units) of unstimulated empty vector control in each experiment. Statistical analysis was performed using two-sided pairwise multiple comparison with Tukey’s adjustment under estimated marginal means (emmmeans) function based on linear mixed-effect models using each individual experiment as a block. Significance is indicated with asterisks: *p < 0.05, **p < 0.01, ***p < 0.001 (empty control p = 0.05, V402M p < 0.0001, R415C p = 0.0006 and R415H p < 0.0001)
Thermostability assays. For peptide and phenyl phosphate experiments, proteins were desalted into 100 mM NaCl, 20 mM Tris (pH 8.0), 2 mM TCEP buffer and diluted to 100 μM. Where peptides were used, a five-fold molar excess of peptide was added to each sample, in phenyl phosphate conditions concentration was 8 mM. For experiments were phosphotyrosine, proteins were diluted to 30 μM in PBS and in phosphate conditions, concentration was 10 mM. Ten microliter of each sample was transferred into a capillary and measured from 35 to 95 °C using a Tycho N67 (Nanotemper). Data were analyzed in Prism.

SPR competition assays. SPR competition assays were performed on either a Biacore 4000 or 200 (GE Healthcare) in 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, 0.1–0.5 μM of WT LNK SH2 domain was pre-incubated with 2, 1, 0.5, 0.25, 0.125 μM of phosphopeptides before being flowed through the chip for 240–720 seconds at 10–30 μl/min. Data was analysed using Prism, the response of LNK in the presence of the peptide was normalised to an LNK only control and then fitted as an I0.5 curve via non-linear regression.

SPR direct binding assay. Direct binding experiments were performed on either a Biacore 4000 or 8000 (GE Healthcare) in 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20 using a streptavidin coated chip and were regenerated in 50 mM NaOH, 1 M NaCl. A biotinylated peptide representing the IL6ST-pY757 sequence was immobilised to the chip by passing over 1 μl/ml of peptide dissolved in 10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20. 0.1–0.5 μM of WT LNK SH2 domain was pre-incubated with 2, 1, 0.5, 0.25, 0.125 μM of phosphopeptides before being flowed through the chip for 240–720 seconds at 10–30 μl/min with immobilised biotinylated JAK2 pY813 bound to determine binding kinetics and affinity. A reference flow cell was included by passing buffer without protein over a single lane and the sensorgrams from the reference cell were subtracted from the experimental flow cell analyses. Data were subsequently plotted in Prism.

Crystallography. All LNK constructs were buffer exchanged into low salt buffer (20 mM Tris (pH 8.0), 2 mM TCEP and 100 mM NaCl) and crystal trays were set up with 5 μg/ml of protein and a 2-fold molar excess of peptide using vapour diffusion sitting drop experiments at the collaborative crystallisation centre, CSIRO (JAK2 pY813) or hanging drops set up in house (EPOR pY454). The WT LNK SH2 domain with the JAK2 pY813 peptide crystalized in 0.2 M ammonium chloride, (JAK2 pY813) or hanging drops set up in house (EPOR pY454). The WT LNK SH2 domain with the JAK2 pY813 peptide crystalized in 20% w/v PEG 8000, 0.05 magnesium acetate, 0.1 M Tris (pH 8.5). All crystals were cryo-protected in paratone and immediately snap frozen in liquid nitrogen. Data was collected at the MX2 beamline at the Australian Synchrotron. Data reduction, scaling and integration was performed using XDS44. Crystal structures of the LNK SH2 domain were solved by molecular replacement (search model PDB ID: 2HDX for WT LNK SH2/JAK2 pY813 structure, and WT LNK SH2 JAK2 activation and downstream signaling pathways.

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Crystalization trials were performed at CSIRO collaborative crystallisation centre (C3).

Author contributions

R.M., Y.Z., J.M.M., A.L. Carried out experiments. R.M., Y.Z., J.M.M., A.L., J.E., C.G.V., N.I.K., J.J.B. designed experiments and analysed and interpreted data. R.M., N.I.K., collected and analysed crystallographic data. R.M., N.I.K., J.J.B. wrote the manuscript. R.M., Y.Z., J.E., J.M.M., N.I.K., and J.J.B. revised the manuscript. J.J.B. Conceived project and designed the study.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information

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