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Structural basis of competition between PINCH1 and PINCH2 for binding to the ankyrin repeat domain of integrin-linked kinase

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ABSTRACT

Formation of a heterotrimeric IPP complex composed of integrin-linked kinase (ILK), the LIM domain protein PINCH, and parvin is important for signaling through integrin adhesion receptors. Mammals possess two PINCH genes that are expressed simultaneously in many tissues. PINCH1 and PINCH2 have overlapping functions and can compensate for one another in many settings; however, isoform-specific functions have been reported and it is proposed that association with a PINCH1- or PINCH2-containing IPP complex may provide a bifurcation point in integrin signaling promoting different cellular responses. Here we report that the LIM1 domains of PINCH1 and PINCH2 directly compete for the same binding site on the ankyrin repeat domain (ARD) of ILK. We determined the 1.9 Å crystal structure of the PINCH2 LIM1 domain complexed with the ARD of ILK, and show that disruption of this interface by point mutagenesis reduces binding *in vitro* and alters localization of PINCH2 in cells. These studies provide further evidence for the role of the PINCH LIM1 domain in association with ILK and highlight direct competition as one mechanism for regulating which PINCH isoform predominates in IPP complexes. Differential regulation of PINCH1 and PINCH2 expression may therefore provide a means for altering cellular integrin signaling pathways.

1. Introduction

The integrin-linked kinase (ILK) is an essential cytoplasmic protein important for signaling to and from integrin adhesion receptors (Legate et al., 2006; Hannigan et al., 2005; Wu, 2005; McDonald et al., 2008). ILK has critical roles in anchorage-dependent cell growth and survival, cell cycle progression, epithelial to mesenchymal transition, cell motility, contractility and early development (Yasunaga et al., 2005; Sakai et al., 2003; Hannigan et al., 2005). ILK is also required for cardiac, vascular, brain, kidney, muscle, skin, platelet, chondrocyte and T cell function and plays important roles in tumor angiogenesis (Legate et al., 2006; McDonald et al., 2008). ILK contains an N-terminal ankyrin repeat domain (ARD), composed of 5 ankyrin repeats (Chiswell et al., 2008; Yang et al., 2009) followed by a predicted kinase domain (Fig. 1A). Genetic analyses in flies, worms, fish and mice show the importance of ILK as a signaling and cytoskeletal scaffold but the kinase activity of ILK remains controversial (Zervas et al., 2001; Postel et al., 2008; Mackinnon et al., 2002; Legate et al., 2006; Sakai et al., 2003). Indeed recent data indicate that ILK kinase activity is dispensable for mouse development (Lange et al., 2009). Numerous ILK binding partners have been identified, including PINCH, parvin, β integrins, paxillin, ILK-associated phosphatase and kindlins (Harburger and Calderwood, 2009; Legate et al., 2006; McDonald et al., 2008).

ILK is normally found in complex with two other proteins: PINCH and parvin (Legate et al., 2006; Wu, 2004), and while ILK kinase activity is not essential for mouse development the formation of a complex with parvin is required (Lange et al., 2009). The heterotrimeric complex between ILK, PINCH and parvin, termed the IPP complex, is an essential signaling platform that regulates cell adhesion, spreading and migration. In mammals, formation of the IPP complex stabilizes expression of the constituent proteins, at least in part by reducing their degradation by the proteosome (Fukuda et al., 2003; Stanchi et al., 2005), and is required for their

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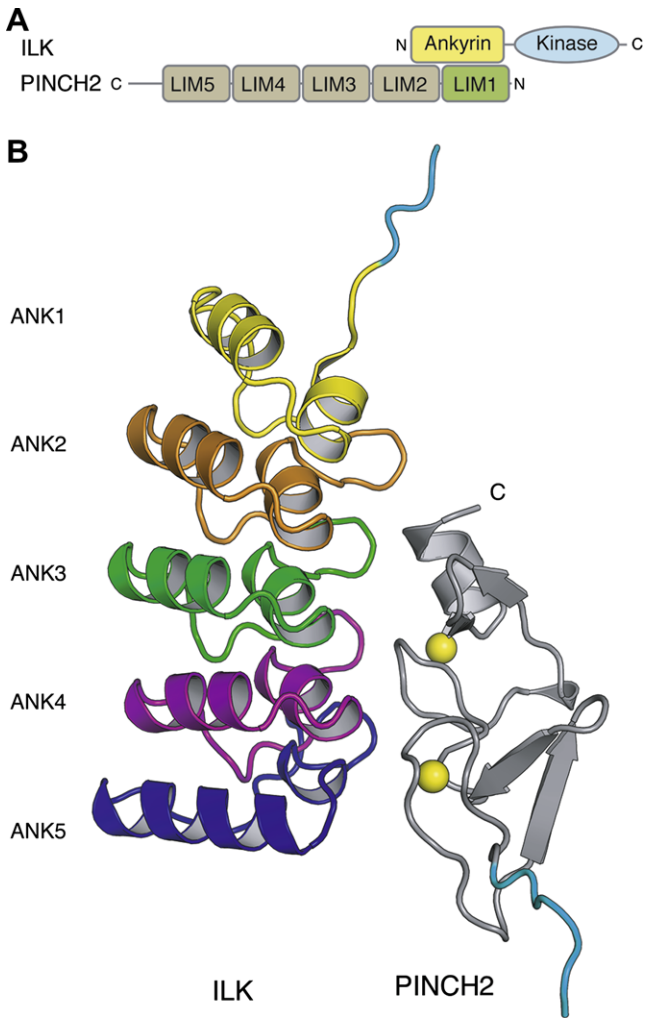

Fig. 1. Structure of PINCH2 LIM1 in complex with ILK ARD. (A) Schematic representation of the domain interactions of ILK and PINCH2. (B) Cartoon of the ILK ARD domain in complex with PINCH2 LIM1. ILK is colored according to ARD repeat (ANK1 yellow, ANK2 red, ANK3 green, ANK4 purple, ANK5 blue). PINCH2 is shown in grey with zinc atoms as yellow spheres. For both proteins, vector derived sequence at the N-terminus is in light blue. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

correct targeting to adhesions (Zhang et al., 2002b; Stanchi et al., 2009). Mammals contain two PINCH proteins, PINCH1 and PINCH2, and three parvins, α-, β- and γ-parvin, thus several different IPP complexes may be formed depending on which combination of PINCH and parvin isoforms bind to ILK. While the physiological significance and specificity determinants of complex formation of these different complexes is poorly understood it is proposed that the signaling specificity of the IPP complex depends on which PINCH isoform it contains (Legate et al., 2006). The formation of different IPP complexes containing PINCH1 or PINCH2 may therefore be a bifurcation point in integrin signaling.

PINCH1 and PINCH2 bind ILK (Braun et al., 2003; Zhang et al., 2002a) and consist of five LIM (Lin11, Isl1 and Mec3) domains followed by a short C-terminal tail. PINCH1 and 2 share 85% sequence identity (Braun et al., 2003; Zhang et al., 2002a) although PINCH2 contains an 11 amino acid extension on the C-terminal tail. PINCH1 is widely expressed throughout development, and PINCH1$^{-/-}$ mice die at the peri-implantation stage with defects in cell–matrix adhesions, cell polarity and cell survival (Li et al., 2005; Liang et al., 2005). In contrast, PINCH2 is expressed later during development than PINCH1 (Braun et al., 2003; Liang et al., 2009; Fukuda et al., 2003) and, possibly due to compensation by up-regulated PINCH1, PINCH2$^{-/-}$ mice exhibit no overt phenotype (Stanchi et al., 2005). PINCH1 and 2 exhibit overlapping expression patterns and in some tissues PINCH2 may compensate for loss of PINCH1 and *vice versa*, suggesting that PINCH1 and PINCH2 have some functional redundancy (Liang et al., 2009, 2005; Stanchi et al., 2005). Consistent with this, ectopic PINCH2 expression can rescue some cellular phenotypes associated with loss of PINCH1 and protects ILK from degradation (Zhang et al., 2002a; Braun et al., 2003; Fukuda et al., 2003; Stanchi et al., 2005). However, in some cell types over-expressed PINCH2 inhibits spreading and migration, possibly by competing with PINCH1 (Zhang et al., 2002a; Shi et al., 2008). The C-terminal tail of PINCH is important for the differential effects (Xu et al., 2005) which are likely to be due to differential binding of accessory proteins to the two PINCH isoforms (Legate et al., 2006; Dougherty et al., 2005). Signaling may therefore depend on which PINCH is present in the IPP complex.

The ILK-binding site in PINCH is localized primarily in the first LIM domain, which interacts with the ARD of ILK (Stanchi et al., 2005; Legate et al., 2006; Tu et al., 1999; Zhang et al., 2002c; Chiswell et al., 2008; Yang et al., 2009) (Fig. 1A). We previously used X-ray crystallography, point mutagenesis and protein–protein interactions studies to reveal the structural basis of ILK–PINCH1 interactions (Chiswell et al., 2008). Here we report the crystal structure of the ILK ARD–PINCH2 LIM1 complex, which shows a striking similarity to the equivalent ILK–PINCH1 complex. Consistent with this, we demonstrate that the LIM1 domains of PINCH1 and PINCH2 compete for binding to ILK. Furthermore, we identify mutations in ILK and PINCH2 that disrupt complex formation *in vitro* and prevent proper localization of ILK/PINCH to integrin-rich focal adhesions in cells. Overall we show that ILK can interact in an experimentally identical manner with a conserved surface on PINCH1 or PINCH2. Thus, the formation of PINCH1- or PINCH2-containing IPP complexes is likely to be largely determined by competition between available levels of PINCH1 or 2.

2. Materials and methods

2.1. Protein expression and purification

An ILK ARD–PINCH2 LIM1 complex was produced and purified using the same strategy previously described for the ILK ARD–PINCH1 LIM complex (Chiswell et al., 2008). Briefly, recombinant GST-tagged human ILK 1–192 (Swiss-Prot Q13418) and His-tagged human PINCH2 6–68 (numbered according to Swiss-Prot Q7Z4I7-3) were produced separately in *Escherichia coli* BL21-Gold(DE3) (Stratagene) using pGEX-4T and a modified pET32 expression vectors respectively. Following induction cells were pelleted, resuspended, mixed together and co-lysed using a freeze–thaw protocol and sonication. Following clarification the complex was affinity purified using the PINCH2 N-terminal 6× His-tag on His-bind resin (Novagen), eluted with 500 mM imidazole and then bound to glutathione-Sepharose 4 Fast Flow medium (Amersham Biosciences) using the N-terminal ILK GST-tag. The glutathione-Sepharose-bound complex was washed and the GST and 6× His-tags were removed simultaneously with thrombin (Enzyme Research Laboratories). Mass spectrometry and N-terminal sequencing revealed there to be an internal thrombin proteolysis of ILK1-192 that generates a fragment spanning ILK1-174 (Chiswell et al., 2008). The cleaved ILK 1–174–PINCH2 6–68 complex was further purified with anion exchange using a MonoQ 5/50 GL column (GE Healthcare) and fractions containing the complex were pooled and concentrated.