The Lnk/SH2B adaptor provides a fail-safe mechanism to establish the Insulin receptor-Chico interaction

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Abstract

Background: Insulin/insulin-like growth factor signalling (IIS) has been described as one of the major pathways involved in growth control and homeostasis in multicellular organisms. Whereas its core components are well established, less is known about the molecular functions of IIS regulators. The adaptor molecule Lnk/SH2B has been implicated in IIS but the mechanism by which it promotes IIS activity has remained enigmatic.

Results: In this study, we analyse genetic and physical interactions among InR, Chico and Lnk in Drosophila tissues. FRET analysis reveals in vivo binding between all three molecules. Genetically, Lnk acts upstream of Chico. We demonstrate that Chico's plasma membrane localisation is ensured by both its PH domain and by the interaction with Lnk. Furthermore, Lnk is able to recruit an intracellular InR fragment to the membrane.

Conclusions: Thus, by acting as a scaffolding molecule that ensures InR and Chico enrichment at the membrane, Lnk provides a fail-safe mechanism for IIS activation.

Background

The Insulin/insulin-like growth factor signalling (IIS) pathway has emerged in the last decade as one of the major signalling pathways involved in the control of growth, body size and homeostasis in multicellular organisms [1-4].

The main intracellular components of IIS in Drosophila are Chico, the homologue of the Insulin Receptor Substrates (IRS), the lipid kinase phosphoinositide 3-kinase (PI3K), the lipid phosphatase PTEN and the serine-threonine kinase dAkt/PKB [5-10]. These intracellular signalling components need to be recruited to the cortical membrane to regulate signalling activity [5,7,11-13]. In addition to the core components, regulators such as Susi [14], Steppke [15] and Lnk [16,17] modulate IIS activity.

The Lnk adaptor protein has been identified in an unbiased screen as a component of the pathway based on the reduced body size and lipid accumulation observed in lnk mutant flies [17]. Mutations in the lnk locus were able to rescue the overgrowth phenotype caused by overexpression of InR, but not to suppress the overgrowth promoted by high activity of PI3K, suggesting that Lnk acts between InR and PI3K in the IIS pathway [17]. Moreover, phosphorylation of PKB and tGPH reporter localisation [18], both readouts of IIS pathway activity, were impaired in lnk mutants [17]. Lnk is the unique Drosophila member of the SH2B protein family. This protein family is characterised by several conserved domains: the N-terminal proline-rich stretch, a pleckstrin homology (PH) domain, a Src homology 2 (SH2) domain, and a C-terminal c-Cbl recognition motif [19-21]. Alleles with inactive PH or SH2 domains have similar phenotypes to those carrying premature stop codons, suggesting that both domains are essential for Lnk activity [17].

Here we study the molecular function of Lnk in Drosophila. We first apply the Förster Resonance Energy Transfer (FRET) technique in Drosophila larvae to demonstrate that Lnk binds to Chico and InR in vivo. Second, we show that Lnk functions upstream of Chico. Finally, we demonstrate that Lnk ensures proper localisation of InR and Chico to trigger IIS.

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Results and discussion

InR, Chico and Lnk physically interact in vivo

Previous studies have demonstrated that a mammalian homologue of Lnk, SH2B, co-immunoprecipitates with the mammalian InR in cultured cells [20,22]. Moreover, Lnk and Chico have been shown to co-immunoprecipitate in Drosophila S2 cells [16]. However, the interactions between the three molecules in vivo have remained elusive. Therefore, we set out to investigate the binding between InR, Chico and Lnk using FRET in Drosophila tissues. We generated constructs to drive expression of tagged InR, Chico and Lnk proteins based on the UAS/Gal4 system [23]. In order to analyse the physical interactions between the three molecules in vivo, we modified phiC31 UASattB vectors to C-terminally tag the expressed proteins with Cyan Fluorescent Protein (CFP) and monomeric Red Fluorescent Protein (RFP), respectively (see Methods).

We first assessed the FRET efficiency (FRETeff) between the known binding partners InR and Chico by overexpressing UAS-InR-CFP and UAS-chico-RFP with hsp-Gal4 in larval salivary glands. We chose the salivary glands because of the large cell size. FRET between CFP and RFP (FRETeff = 14.1 ± 3%) was observed in 71% of the tissue samples examined after insulin stimulation (Figure 1A and F). Interestingly, we detected FRET between the two molecules only in 20% of the samples in the absence of insulin (FRETeff = 13.5 ± 1%), indicating that InR-Chico binding is stimulated by insulin as previously reported [24,25].

We next investigated the binding of Lnk to Chico. In Lnk-CFP/chico-RFP salivary glands, FRETeff was 14.8 ± 4% in 73% of the samples upon insulin stimulation (Figure 1B and F). By contrast, non-stimulated tissue samples showed a reduction in Lnk-Chico interaction (FRETeff = 13.3 ± 3% in 30% of samples), suggesting that the plasma membrane, reflecting high IIS activity (Figure 2F, [17]). By contrast, overexpression of chico-RFP in lnk mutant salivary glands resulted in localisation of the tGPH reporter to the plasma membrane, reflecting high IIS activity (Figure 2E and G). Thus, overexpression of chico-RFP counteracts the loss of lnk function, suggesting that Chico acts downstream of Lnk.

To analyse whether Lnk facilitates the localisation of Chico, we first studied the localisation of Chico-RFP in lnk mutant salivary glands. We determined the intensity of the Chico-RFP signal at the membrane and in the cytoplasm to assess the relative amounts of protein in these compartments under different experimental conditions (see Methods). The membrane localisation of Chico-RFP was only slightly reduced in lnk mutant tissue in comparison to wild-type tissue (Figure 2H, I and Additional file 1: Figure S1A), probably due to the PH domain of Chico. In fact, when we expressed a PH-domain mutated form of Chico (Chico-PH*-RFP) in a lnk mutant background, we noticed a relocalisation of Chico-PH*-RFP from the membrane to the cytoplasm (Figure 2K and Additional file 1: Figure S1A). By contrast, Chico-PH*-RFP showed significant localisation to the plasma membrane in wild-type tissue (Figure 2J and Additional file 1: Figure S1A), indicating that Lnk is sufficient to substitute the function of the PH domain in Chico-PH*-RFP. Thus, Lnk provides a redundant means to properly localise Chico at the cortical membrane.
Figure 1 In vivo FRET analysis reveals physical interactions among Lnk, Chico and InR. (A-A’) Chico-RFP (A’) and InR-CFP (A) strongly co-localise in salivary glands (A’). FRET° shows regions with high energy transfer between CFP and RFP (colour code: black - low intensities, red - high intensities). (B-B’) Lnk-CFP (B) and Chico-RFP (B’) co-localise (B’) showing positive FRET° (B’). (C-C’) InR-CFP (C) and Lnk-RFP (C’) localisation (C’) and FRET (C”) at the cortical membrane. (D-D’) InR-CFP (D) and DI-RFP (D’) do not exhibit energy transfer (D”). (E-E’) Lnk-CFP (E) and DI-RFP (E’) do not show positive FRET° (E”). (F) Table showing data from FRET analyses. Number of samples analysed: (A) n = 21 (+ins) and n = 10 (−ins), (B) n = 20 (+ins) and n = 10 (−ins), (C) n = 17 (+ins) and n = 11 (−ins), (D) n = 15, (E) n = 14. Scale bars represent 50 μm.
Figure 2 (See legend on next page.)
Lnk ensures InR enrichment at the cortical membrane

Our genetic and localisation data of Chico and Lnk might seem contradictory to previous genetic interaction experiments between chico and lnk mutants; if Lnk was only required for proper Chico function, chico; lnk double mutants should display similar phenotypes to chico single mutants. However, whereas the single mutants are reduced in size but viable, the chico; lnk double mutants turned out to be lethal [17]. One way to reconcile these findings is to propose an additional direct function of Lnk on InR.

We analysed InR-CFP localisation in lnk mutant salivary glands to test whether Lnk facilitates InR localisation. In contrast to InR-CFP in wild-type tissue, where InR-CFP was located mainly at the cortical membrane (Figure 3A and Additional file 1: Figure S1B), InR-CFP was decreased at the membrane in a lnk mutant background (Figure 3B and Additional file 1: Figure S1B). However, a fraction of InR-CFP still localised at the plasma membrane, most likely due to InR’s transmembrane domain. We next generated an intracellular InR construct (hereafter InR(INTRA)) containing the intracellular domain of InR (beginning with the kinase domain) fused to CFP at the C-terminus (InR(INTRA)-CFP). InR(INTRA)-CFP membrane localisation was reduced already in a wild-type background (Figure 3C and Additional file 1: Figure S1B), whereas full-length InR-CFP showed a similar effect only in lnk mutant salivary glands. In a lnk mutant background, cortical accumulation of InR(INTRA)-CFP was reduced more strongly (Figure 3D and Additional file 1: Figure S1B), further supporting the role of Lnk in locking InR to the membrane. Moreover, overexpression of InR(INTRA)-CFP together with lnk-RFP restored cortical localisation of InR(INTRA)-CFP, showing both molecules at the plasma membrane (Figure 3E and Additional file 1: Figure S1B). The membrane localisation was essentially abolished when a PH domain mutant version of Lnk was expressed (Figure 3F and Additional file 1: Figure S1B). These experiments strongly suggest that Lnk contributes to the cortical localisation of InR by interacting with the intracellular part of InR.

Finally, we investigated the interaction between InR-CFP and Chico-RFP in a lnk mutant background. Similarly to Lnk, Chico-RFP was able to recruit InR(INTRA)-CFP to the membrane, either in a wild-type (Figure 4A and Additional file 1: Figure S1C) or in a lnk mutant (Figure 4B and Additional file 1: Figure S1C) background, although a significant proportion of InR(INTRA)-CFP and Chico-RFP remained in the cytoplasm when Lnk was lacking. However, when InR(INTRA)-CFP and Chico-RFP were overexpressed in lnk mutant salivary glands, the membrane enrichment of both was abolished (Figure 4D and Additional file 1: Figure S1C) in contrast to the situation where Lnk was present (Figure 4C and Additional file 1: Figure S1C). Thus, Lnk is required to reinforce the InR-Chico interaction at the membrane.

Conclusions

By combining genetics with in vivo localisation studies on InR and its two adaptor molecules, Chico and Lnk, we gained insight into the molecular mechanisms at the plasma membrane that ensure proper IIS activation. Our data support the following model: Lnk is required to enrich InR and Chico at the plasma membrane (Figure 4). In a lnk mutant situation, fractions of InR and Chico are still localised at the cortical membrane, due to their transmembrane and PH domains, respectively. Thus, the IIS pathway is partially active in the absence of Lnk (Figure 4E). In chico mutants, InR is capable of directly interacting with PI3K [28]; hence chico mutants are viable (Figure 4E). By contrast, chico lnk double mutants are lethal, probably due to mislocalisation and/or instability of InR, resulting in an insufficient signal from the receptor to PI3K (Figure 4E). Mutations in lnk weaken the InR-Chico interaction, reducing the capability of InR to phosphorylate Chico, as it was observed by Song and colleagues [16]. Future studies should aim at elucidating the precise mechanism of how Lnk promotes the InR-Chico interaction. It remains to be determined whether Lnk is required for trafficking, localisation at specific subdomains of the cortical membrane or stabilisation of InR and/or Chico.
Methods
Lnk, chico and InR constructs
PCRs from Drosophila *lnk*, *chico* and *InR* genes were performed using primers listed in Additional file 2: Table S1. Point mutations introduced in the PH domains of Lnk and Chico were C254Y and W100L, respectively [17,29]. PCR products were cloned into pENTR-TOPO (Invitrogen). Subsequent Gateway reactions were performed to shuffle the sequences into modified pUASTattbCFP and pUASTattbRFP vectors.
Figure 4 (See legend on next page.)
The pUASTatb vector was modified in order to introduce CFP or RFP coding sequences. pUASTatb and pAWC or pAWR (obtained from Drosophila Genomics Resource Center) were digested with NheI and NotI to swap the Gateway cassette from the pUASatb plasmid to pAWC or pAWR cassettes, respectively, to introduce CFP or mRFP coding sequences downstream of the attR2 sites.

**Fly transgenes and mutants**

Mutant alleles used were *Ink*4Q3 [17] and *chico*1, *hsp-Gal4* and GMR-Gal4 [30] were used to drive expression of the transgenes. Vectors carrying LIAS-Inr, Inr**INTRA**, *chico* or *Ink* were injected in ZH-attP-86Fb or ZH-attP-44F (Inr**INTRA**) embryos [31]. The functionality of the tagged proteins was confirmed as follows (Additional file 3: Figure S2): Overexpression of *Lias-Inr-CFP* using the GMR-Gal4 driver [30] resulted in a significant overgrowth of the eyes as compared to GMR-Gal4 *Lias-IGF* control flies. Overexpression of *Lias-Chico-RFP* and *Lias-Inr-CFP* (by means of MARCM clones) in eye imaginal discs promoted higher phospho-PKB levels (as a readout for IIS activity). The *tGPH* line was used as a reporter for IIS activity [18]. *Lias-Delta-RFP* (Bloomington 26696) [27] was used to perform the negative controls for the FRET experiments.

**Sample processing, FRET analyses and quantification of sublocalisation**

Transgenic lines were crossed to *hsp-Gal4*. Third instar larvae were incubated at 37°C for 1 h. After 45 min of recovery, salivary glands were dissected in PBS and incubated for 15 min in Schneider’s medium with or without insulin (100 nM). After insulin treatment, salivary glands were fixed in 4% paraformaldehyde.

To examine interactions between Lnk, Chico and InR proteins, the FRET’sensitised emission method was used. CFP was utilised as donor molecule and mRFP as acceptor molecule. FRET was analysed using a Leica SP2-AOBS confocal microscope. The FRET values were corrected for background fluorescence and crossover of donor and acceptor fluorescence. Corrected FRET was calculated as $FRETc = FRET - (a \times CFP) - (b \times RFP)/RFP$, with a and b representing the fractions of bleed-through of CFP and RFP fluorescence, respectively, through the FRET filter channel [32]. These values are presented as FRET efficiency (FRETeff). FRETeff values were averaged from regions of interest (ROIs) observed in cells from three independent experiments (n > 10) per condition and represented as mean ± standard deviation. For our sensors, we considered FRETeff ≥10% as positive FRET [33-35]. However, it is generally accepted that absence of FRET yields values <3%. To determine the percentage of samples (tissues) with positive FRET, we considered any ROI with FRETeff ≥10% after scanning regions where the two constructs co-localised as positive. After applying Gaussian Blur filter (Sigma: 2) with Imagej software, FRET was presented in pseudocolour mode according to a temperature-based Look Up Table (LUT) with blue (cold) indicating low values and red (hot) indicating high values. LUT was linear, covering the full range of the data.

To quantify the subcellular distribution of the tagged proteins, the Plot Profile function from Imagej software was used. Pixel intensities at the membrane and in the cytoplasm were measured across the cell (at least two cells were measured in each tissue sample), avoiding the nuclei and taking Actin pixel intensity as indicator for the membrane sub-compartment. Pixel intensities were averaged for each fraction, and the ratio between the means was calculated as a measure for the relative amount of protein per sub-compartment. The R package was used to perform one-tail t-test statistical analyses and boxplots.

**Immunostainings and clonal analysis**

For dominantly marked clones (MARCM system), *FRT40 chico*1 or *FRT40iso* and *y w hs-flp Lias-GFP; tub-Gal80 FRT40/CyO y*; *tub-Gal4/TM6B flies* were used. Clones were induced in second instar larvae (48 h after egg deposition (AED)) at 37°C for 15 min (dissection 96 h AED). Rabbit anti *Drosophila* phospho-Akt/PKB Ser505 (1:300, Cell Signaling) staining was carried out on eye imaginal discs. Discs were dissected in PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. After blocking (PBS, 0.3% Triton X-100, 2% NDS), imaginal discs were incubated with primary antibody at 4°C overnight. Goat anti-rabbit-Cy3 (1:200, Molecular Probes) was used as secondary antibody for 2 h at 4°C.
room temperature. AlexaFluor 647 phalloidin (1:40, Molecular Probes) was used for Actin staining. Nuclei were stained with DAPI before mounting in Vectashield. Samples were captured using a Leica SPE TCS confocal laser scanning microscope. Images were processed using NIH ImageJ software. Final artwork was prepared using Adobe Photoshop CS5 and Illustrator.

Additional files

Additional file 1: Figure S1. Summary of subcellular localisation analyses. (A) Boxplots and data summary related to Figure 2. (B) Boxplots and data summary corresponding to Figure 4.

Additional file 2: Table S1. Additional file 3: Figure S2. InR-CFP, Chico-RFP and Lnk-CFP fusion proteins promote IIS activity. [A–C] Overexpression of UAS-InR-CFP using the GMR-Gal4 driver (B) results in overgrown eyes as compared to the control (UAS-GFP, A). (C) Eyes of GMR > InR-CFP and GMR > GFP flies (n = 13, p < 0.01) (D–D”) MARCM clones in eye discs overexpressing UAS-Chico-RFP show an increase in phospho-PKB levels (D”) and (D’). (E–E”) MARCM clones overexpressing Lnk-CFP (E”) exhibit increased phospho-PKB levels (E’”) and (E”). Scale bars represent 100 μm (A and B) and 50 μm (D and E), respectively.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
IA, EH and HS conceived the experiments. IA and IP performed the experiments. IA analysed the data. IA and HS wrote the paper. All the authors read and approved the final manuscript.

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