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Structure of Lipid Kinase p110 b/p85b Elucidates an Unusual SH2-Domain-Mediated Inhibitory Mechanism

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SUMMARY

basis for the unanticipated inhibition by the cSH2, we determined the crystal structure of the p110 b catalytic subunit in a complex

We compared the catalytic activity of puri ed recombinant proteins using either a high-throughput uorescence polarization assay, which measures the production of ADP (Klink et al., 2008) (Supplemental Experimental Procedures), or assays that measure PIP_3 production directly (using radioactive assays and mass spectroscopy). The activity of the p110 b catalytic subunit with the lipid substrate diC8-PIP_2/POPS is strongly inhibited by a p50-like regulatory subunit core that contains both the nSH2 and cSH2, linked through the iSH2 (nicSH2) (Figure 1B). The basal activity of the p110b/p85 b-nicSH2 is considerably lower than a complex containing only the iSH2 or a construct of the catalytic subunit without the regulatory subunit, DABD-p110 b. We used DABD-p110 b as a substitute for the free full-length catalytic subunit, since the latter cannot be stably expressed in insect cells (Berndt et al., 2010).

To dissect the regulatory roles of each of the three domains present in the p85 b-nicSH2 construct, we assayed further truncation variants of p85 b for their ability to inhibit the basal activity of p110b. Our results clearly show that both nSH2 and cSH2 contribute signi cantly to inhibition of p110 b. Each SH2 domain inhibits the enzyme relative to the complex containing only iSH2 (Figure 1B). Compared to the complex with both SH2 domains (nicSH2), the absence of either the nSH2 or the cSH2 leads to enzyme activation as measured by ADP formation (Figure 1B) or by PIP_3 5 (PD58(735)(a)–7 5 whnnot3

This complex shows that both the iSH2 and cSH2 are ordered and interact with the p110 b catalytic subunit. In the overall arrangement of the two subunits, the long coiled coil of the iSH2 slots into the large arch formed by the catalytic subunit, while the cSH2 nestles against the C-terminal region of the kinase domain (Figure 2A). The structure of the complex reveals possible underlying mechanisms of inhibition by the cSH2. In contrast to the nSH2 of p85 a, which is in contact with three domains of p110 a (C2, helical, and kinase domains) (Mandelker et al., 2009; Miled et al., 2007), the cSH2 of p85b contacts only the C lobe of the p110 b kinase domain. The solvent accessible surface area buried by the cSH2/p110 b interface (1264 A^2) is smaller than the nSH2/p110a interface (1793 A^2). Our structure shows that a cSH2 loop (Ala674 to Tyr680), which was described as a protrusion at the surface of cSH2 (Hoedemaeker et al., 1999), forms the main contact point with the p110b. This loop interacts with a double layer of helical pairs, the Ka7/Ka8 and the C-terminal Ka11/Ka12 of the kinase domain (Figures 2

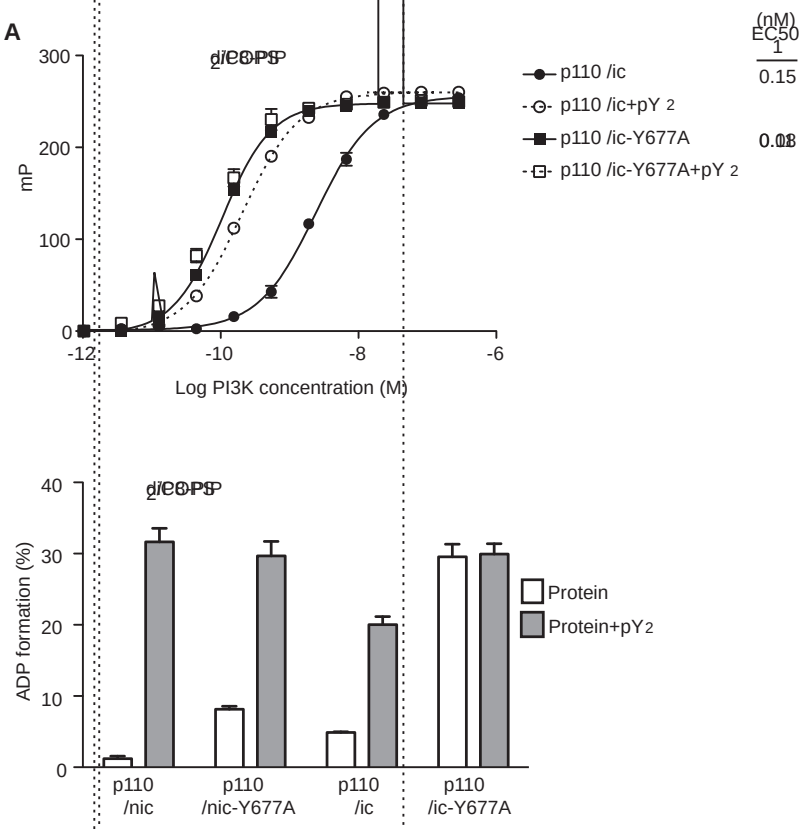

Figure 3. Mutation Y677A in p85 b-cSH2 Releases

(A) Kinase activity (ADP formation) of p110b in a complex

(ADP formation on the y axis expressed as in Figure 1B).

(B) Comparison of p110b activities of 1 nM complexes with wild-type or Y677 mutant nicSH2 or icSH2 (activity shown as in Figure 1D).

Inhibitory Effect on p110 b

Effect of Contact Mutation on Basal Activity and RTK Activation

with p85b-icSH2-Y677A (Y677
wild-type complex (ic) in the ab
the PDGFR pY

The structure of the PI3Kb complex has identified Tyr677 in the p85 b cSH2 as making contact with the elbow region of the p110 b. We mutated Tyr677 to alanine to see if this contact regulates p110 b activity. The mutant p110b/p85 b-icSH2-Y677A complex in the absence of pY_2 is more active than the wild-type p110b/p85 b-icSH2, and it is not activated by pY_2 (Figure 3A). Similarly, the p110b complex with nicSH2-Y677A, a regulatory construct containing wild-type nSH2 but mutant cSH2-Y677A, has a basal activity higher than the wild-type (Figure 3B), demonstrating that the inhibitory grip of the cSH2 on p110 b can be released by a single mutation in the cSH2. Mutation of another residue, E672A, from the AlaGluPro sequence preceding Tyr677, partially relieves cSH2-mediated inhibition (Figure S3F), consistent with this residue forming part of the interface with the p110b subunit. Alanine from the AlaGluPro sequence in p85a was found mutated in a colorectal cancer (A682V in p85a) (Jaiswal et al., 2009). This mutation slightly activates p110b (Figure S3

Figure S3C. offerJaTJ-15227.6.24hl(032-m5esng

immediately how phosphopeptide binding releases nSH2 inhibitory effects on p110 a (Mandelker et al., 2009). In contrast, the pY-binding site on the cSH2 is exposed and not buried in the interface with the p110b (Figure 2D). Superposition of the cSH2 bound to a PDGFR phosphopeptide (PDB ID: 1H9O) (Pauptit et al., 2001) suggests that a peptide should have more than four residues following the pY in order to break the cSH2/p110 b contact. Indeed, we found that three different RTK phosphopeptides with seven residues following the pY (pY + 7) activated the p110b/p85 b-icSH2 complex, whereas pY + 4 peptides failed to activate this complex (Figure 2E). Nevertheless, all six peptides activated the p110 b/p85 b-niSH2 complex to a similar level. These results suggest that the fundamental differences between regulation by p85 nSH2 and cSH2 may confer much greater contextual speci city of PI3K activation by RTKs than had been anticipated. Further mapping of PDGFR-derived phosphopeptides shows that inhibition of p110 b/p85 b-icSH2 is relieved by pY + 5 or longer phosphopeptides (Figure S3D). In addition, relief of inhibition by pY + 5 peptides is dependent on the residue identity at the +5 position (Figure S3D). The longer phosphopeptides (pY + 5 to pY + 7) are more ef cient at disinhibiting the enzyme, despite the fact that they have lower af nity for the p110 b/p85b complex compared to the short phosphopeptide (pY + 4) (Figure S3E). This is consistent with the longer peptides having to compete with p110 b for binding to the cSH2, whereas the pY + 4 peptide binds to the cSH2 without displacing the p110b.

activation loop (residues 930–955), although the side-chain density is poor. This loop is responsible for substrate speci city (Bondeva et al., 1998). Our structure shows that the long activation loop extends all the way to the iSH2, resulting in the close vicinity of Asp455 in the iSH2-p85 b (Asp464-p85a) with both Phe943 and Lys942-p110b (Figure 6A). This interaction could potentially restrict the exibility of the activation loop and thereby contribute to inhibiting the enzyme. Mutation D464H-p85 a, which was found in glioblastoma (Parsons et al., 2008), could affect interaction with Phe943 and Lys942, thereby releasing the grip of iSH2 on the activation loop.

In addition to the activation loop, p85-iSH2 contacts the ABD and C2 domains of p110 b differently from the p110 a/p85 a complex (Figures 6B and 6C). Previous results for p110a have shown unambiguously that the iSH2/C2 contact is inhibitory (Wu et al., 2009). Two of the contact areas involve iSH2 residues that are mutated in p85 a subunit in glioblastomas and were shown to activate p110 a, p110b, and p110d

this difference. The third brake is provided by the iSH2, which nestles under the arch formed by the catalytic subunit and forms inhibitory contacts with the C2 domain (Wu et al., 2009). Its mutations in several types of tumors lead to dramatic upregulation of all class IA isozymes. While p110b activity is inhibited by the engagement of three brakes, p110a appears to have only two brakes (nSH2 and iSH2), suggesting that p110a is poised to have greater activity when one of the brakes is lost by mutation. The inhibition of p110b by both SH2 domains of the p85 subunit may explain observations that p110 b is less responsive to RTK stimulation in some cells (Kurosu et al., 1997; Maier et al., 1999; Guillermet-Guibert et al., 2008).

The inhibitory effect of the cSH2 could be explained by its contacts with the C-terminal region of the kinase domain. In our crystal structure, the kinase domain of the catalytic subunit exhibits the signatures of an inactive conformation. The contacts of the cSH2 with the regulatory elements surrounding the activation and catalytic loops, i.e., helices K a11/Ka12 and Ka7/Ka8 forming the double-layer regulatory arm, could possibly pin the catalytic and activation loops into an inactive conformation and prevent the C-terminal helix from swinging out to its active conformation. By affecting the C-terminal helix conformation, the cSH2 may also affect membrane binding. In the primordial class III PI3K Vps34, the equivalent C-terminal helix interacts with membranes and its deletion abolishes enzyme activity (Miller et al., 2010). Similarly, we show here that the C terminus in p110b is critical for lipid kinase activity.

Interestingly, the primary interaction site of p110 b with the cSH2 is part of a regulatory “square” composed of helices Ka10, Ka11, and Ka12, which were described as three sides of an imaginary rectangle (

bK

Overexpression of wild-type p110 b

EXPERIMENTAL PROCEDURES

Constructs, Design and Cloning

Plasmids encoding p110b, p85a, p85b, and p55g constructs shown in Table S2 were generated using the In-Fusion PCR method (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). The p110b constructs cloned into pFastBac-HTb (Invitrogen Ltd, Paisley, UK) have an N-terminal extension encoded by the vector (MSYHHHHHHDYDIPTTENLYFQGAMDL), comprising a His_6 tag and a TEV-protease cleavage site. The p85 and p55 constructs cloned into pFastBac1 (Invitrogen) do not have tags. Point mutations in p110b, p85, and p55 constructs shown in Table S2 were generated using the Quick-Change protocol (Agilent Technologies UK Ltd., Stockport, Cheshire, UK) and veri ed by sequencing.

For mammalian expression, pMIG- and pMIR-derived vectors were used (Kulathu et al., 2008). A myc-tag was inserted at the N terminus of human p110b and a FLAG-tag was inserted at the N terminus of human p85a , using standard PCR and cloning strategy (sequences MEQKLISEEDLGGSTR and MGDYKDDDDKGGSTR ahead of genes). Mutagenesis in p85a was performed in pFastBac1 vectors and subcloned into mammalian expression vectors.

Protein Expression and Puri Þcation

A detailed procedure of protein puri cation is described in the Supplemental Experimental Procedures. Brie y, proteins were expressed in Sf9 cells, using recombinant baculoviruses. Cells were coinfected for 63 hr with viruses encoding the catalytic and regulatory subunits. Cells were lysed by sonication and the protein complexes were puri ed by sequential chromatography on HisTrap, Q-Sepharose, heparin, and gel- ltration columns.

Crystallization

Mouse His_6-p110b(1-1064)/p85b-icSH2(423-722) complex was diluted to 4 mg/ml, mixed with 20 mM (nal concentration) sodium phenyl phosphate (Sigma P-7751) and 150 mM of the PI3K inhibitor GDC0941 (Folkes et al., 2008). The initial crystallization conditions were obtained from a broad screen of 1056 conditions (Stock et al., 2005) in 96-well MRC crystallization plates (SWISSCI AG, Zug, Switzerland). Additives (GDC0941 and phenyl phosphate) were identi ed by differential scanning uorimetry (see Supplemental Experimental Procedures). Optimal crystals were obtained at 22 C in hanging drops over reservoirs of 24-well plates (Hampton Research, Aliso Viejo, CA) containing 12% polyethylene glycol 3350, 0.1 M potassium citrate at pH 6, and 0.4 M lithium sulfate. The drops contained 1 ml each of protein and reservoir solutions. The crystals were cryoprotected by stepwise addition of cryoprotectants consisting of the reservoir solution with 20 mM sodium phenyl phosphate, 150 mM of GDC0941, and an increasing concentration of glycerol up to 20% (in 5% increments). Crystals were ash frozen in liquid nitrogen.

Data Collection and Structure Determination

See Supplemental Experimental Procedures.

Kinase Assays

See Supplemental Experimental Procedures.

Mammalian Cell Culture and Western Blots

See Supplemental Experimental Procedures.

ACCESSION NUMBERS

Coordinates and structure factor amplitudes for the p110 b/p85b crystal structure have been deposited in the Protein Data Bank under ID code 2y3a (Table 1).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, seven gures, two tables, and two movies and can be found with this article online at doi:10.1016/j.molcel.2011.01.026.

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