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A Measured Approach - Determining the PLC γ 1 Docking Site on Itk using a Biochemical Ruler

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In this issue of the *Journal of Molecular Biology* Xie et. al. describe the interaction between IL-2 inducible T-cell kinase (Itk), a non-receptor tyrosine kinase, and its substrate PLC γ 1. The authors demonstrate that a previously determined set of basic amino acid residues¹ on the C-terminal SH2 domain of PLC γ 1, interacts with an acidic patch on the G helix (α G) of the catalytic domain of Itk. The results described here reinforce the importance of α G as a mediator of intermolecular interactions in protein kinases.

PLC γ 1 Docking on Itk: A Non-canonical SH2 Domain Interaction

PLC γ 1 is a substrate of Itk, a member of the Tec family of non-receptor protein tyrosine kinases. Phosphorylation of PLC γ 1 on a specific tyrosine residue (Y783) in T-cells leads to an up-regulation of its lipase activity, resulting in the generation of two second messengers, diacyl-glycerol and inositol triphosphate, ultimately leading to the activation of protein kinase C (PKC).

Determination of the interface between Itk and PLC γ 1, and indeed between any kinase and its substrate, can be a tricky proposition. Not surprisingly, few crystal structures of a kinase/substrate complex are available in the PDB. The alternative, solution NMR, is also not a simple option, since these studies on kinases themselves are usually technically complex and require a significant investment both in time and resources. The larger sizes of kinase-substrate complexes exacerbate these issues further. Hydrogen/deuterium exchange mass-spectroscopy (HXMS)² to probe increased protection at the protein-protein interface in comparison to the corresponding free species, could be a viable alternative. However, changes in dynamics and allosteric effects away from the interaction site could complicate the interpretation of HXMS data. Allosteric effects also complicate the interpretation of NMR-based assays of protein-protein interactions if only chemical shift perturbations are used to infer the interaction surface. In all cases, whatever the method used, crystallography, NMR or HXMS, the validity of the biophysically determined interface should be confirmed using rational site-directed mutagenesis. In the study reported here, Xie *et. al.* skip the cumbersome biophysical steps and instead use a clever biochemical approach to determine the PLC γ 1 docking site on the catalytic domain of Itk (Fig. 1). It was known from previous

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studies, that Itk recognizes a set of basic residues located on the C-terminal SH2 (SH2C) domain of PLC γ 1¹. This docking interaction, that does not involve the canonical phosphotyrosine recognition by SH2 domains, facilitates the phosphorylation of Y783 that is attached to the C-terminus of SH2C by a flexible 29-residue linker. The authors surmised that if this linker were shortened enough, the modifiable Y783 would no longer be able to reach the Itk active site, and thus would not be phosphorylated, since the PLC γ 1 SH2C docking site on the Itk catalytic domain would be too far away. At first, in order to obtain a rough length measurement the authors created two PLC γ 1 constructs in which the linker between SH2C and Y783 was shortened by 20-residues in one (L9), and by 25-residues in the other (L4). Upon interrogating the ability of these constructs to be phosphorylated by Itk, only the L9 construct produced a positive result. Next the authors refined the length obtained from their molecular ruler approach by making two more constructs in which the L4 construct was lengthened by one (L5) or three (L7) residues. Choice of a single glycine (L5) or a glycine-serine-glycine tripeptide (L7) ensured that the engineered linker remained flexible. It turned out that only the L7 construct was efficiently phosphorylated by Itk, thus establishing that the SH2C-docking site was no more than 28 Å (the length of a fully extended linker) away from the Itk catalytic site. Armed with this distance information, and the knowledge that the PLC γ 1 SH2C docking site on the catalytic domain of Itk was most likely to involve acidic residues to complement the known basic cluster on PLC γ 1 SH2C, the authors identified a set of 12 solvent exposed Glu and Asp residues situated within 28 Å of the catalytic site, distributed over both the N- and C-lobes of the Itk catalytic domain, as possible foci for PLC γ 1/Itk interactions. Systematic mutations of these residues combined with enzymatic assays of Itk catalyzed Y783 phosphorylation revealed that the PLC γ 1 SH2C docking site involves a cluster of acidic residues on helix α G on the catalytic domain of Itk.

α G: A Docking Site Extraordinaire

The overall structure of the catalytic domain in eukaryotic kinases is highly conserved as are several structural motifs therein³. Among these structural elements is helix α G that is located on the larger C-terminal lobe of the catalytic domain and has been shown to be a focal point in mediating molecular interactions in several protein kinases. In the p21-activated protein kinase, PAK1, intra-molecular interactions between the catalytic domain and the regulatory inhibitory switch (IS) domain, centered about α G, maintains the kinase in an inactive state⁴. A similar α G-centric inhibitory interaction is also seen between the catalytic domain of protein kinase A (PKA) and its regulatory RI α subunit⁵. In the RNA-dependent protein kinase R (PKR), α G is longer than normal and occupies a non-canonical spatial position⁶. It appears that these unique features are required to optimally position a substrate, the translation initiation factor eIF2 α containing a modifiable serine, near the catalytic cleft. This phosphorylation event that follows viral RNA binding, reduces translation and constitutes a key step in fighting the viral invasion. In MAP kinases, the so-called F-recognition site (FRS) also includes α G, and recognizes sequences containing an FxFP motif (F-site) that is found in many substrates e.g. Elk-1, c-Fos². NMR and biochemical evidence suggests that this site can also bind MAP kinase interacting proteins that do not contain a canonical F-site sequence. Examples include the transcription factor, Ets-1 and the adaptor protein, PEA-15 both of which interact with the MAP kinase ERK2⁷. α G mediated interactions have also been noted in eukaryotic-like serine/threonine kinases that are found in bacteria. In PknB, a serine/threonine kinase from *Mycobacterium tuberculosis*, docking interactions involving the α G helices of both enzyme-acting and substrate-acting molecules leads to trans-phosphorylation (on the activation loop) and kinase activation⁸.

While most protein-protein interactions mediated by α G identified so far largely involve hydrophobic residues with some stabilizing electrostatic interactions, the interaction between the Itk α G and PLC γ 1 SH2C seems to be dominated by charge-mediated interactions, making it quite unique. This is in addition to the novelty in that it involves an SH2-domain interaction that does not entail the recognition of a phosphotyrosine moiety. The precise structural contribution of each α G residue from the Itk catalytic domain and their interactions with the corresponding residues on the PLC γ 1 SH2C can only be ascertained upon the availability of the crystal structure of the complex or perhaps an NMR-data driven structural model. Nevertheless, Xie *et al.*, in addition to illustrating another example of the α G helix as a chameleonic modulator of protein-protein interactions involving kinases, have provided a nifty biochemical trick that can be used to determine protein interfaces when limited structural data is available. This biochemical approach is valid as long as the structures of the participating partners, for which high-resolution structures are available individually, are not significantly altered upon interaction.

It was previously shown that Itk uses a site distinct from the one described here to dock its own SH2 domain, facilitating the phosphorylation of yet another tyrosine (Y180) on its SH3 domain⁹. The use of distinct docking sites to modulate substrate specificity is well known for the MAP kinases. The work of Xie *et al.* lends credence to the possibility that use of multiple docking sites may be a general mechanism employed by protein kinases to distinguish between and process multiple substrates.

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Fig. 1.

Two-step procedure to determine the PLC γ 1 SH2C docking site on Itk. The search for the docking site of the C-terminal SH2 domain of PLC γ 1 (SH2C) on the catalytic domain of Itk was carried out in two steps: In **step 1**, the minimal linker length to allow the relevant tyrosine (Y783) on PLC γ 1 to be successfully phosphorylated (indicated by the red circle in the insets), was determined. D1 is the distance between the docking site (D, green oval) and the catalytic site (C, red oval) on the Itk catalytic domain; D2 is the length of the linker (when fully extended) connecting Y783 to the C-terminus of PLC γ 1 SH2C; successful phosphorylation should take place as long as $D2 > D1$, in the absence of any unforeseen steric inhibition. A minimal linker length of seven residues was found to be required for phosphorylation ($D2_{\min} \sim D1$, panel E for **step 1**) suggesting that the docking site was within about 28 Å of the catalytic site. In **step 2**, multiple patches of negatively charged residues (red ovals), to complement the previously-determined positively charged patch on PLC γ 1 SH2C (indicated by a blue oval), on the Itk catalytic domain within 28 Å of its active site were determined, and their role in docking (mutations at the correct docking site would prevent binding and consequently phosphorylation) were tested through mutagenesis. This two-step procedure revealed that the PLC γ 1 SH2C docking site on the catalytic domain of Itk involved helix α G (panel E for **step 2**), a structural module that is a known hub for molecular interactions in protein kinases.