The Role of the Regulatory Spine in Eukaryotic Protein Kinase Activity

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Protein kinases are highly regulated enzymes that phosphorylate a serine, threonine, or tyrosine in about 30% of human proteins and therefore, are able to regulate several cellular and metabolic processes [1]. There are over 500 Eukaryotic Protein Kinases (EPK) identified to date that are divided into seven subfamilies based on their sequence and function. All EPKS have a highly conserved core that consists of two lobes [2]: a small N-terminal lobe (N-lobe) and a larger C-terminal lobe (C-lobe). The EPK core is organized around three major elements: a large hydrophobic αF-helix in the middle of the C-lobe and two hydrophobic ensembles termed "spines": the Regulatory (R) spine and the Catalytic (C) spine [3,4]. The accurately aligned R-spine is a non-linear structural motif that connects the N- and C-lobes in active EPKs. Recent experiments have demonstrated that disrupting mutations made to the R-spine of the constitutively active cAMP dependent protein kinase (PKA) affect the kinase activity. PKA is used as a model to understand the role of each R-spine residues in the kinase activity of all EPKs.

The Architecture of the Kinase Core

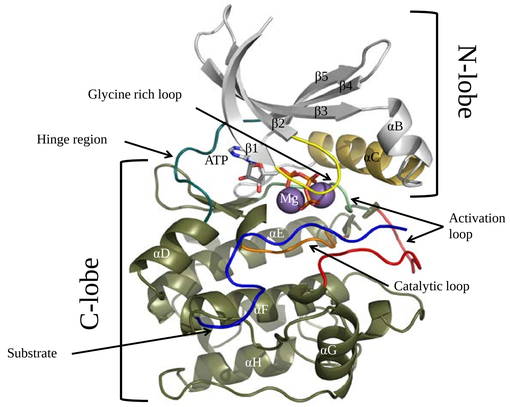

Figure 1: The catalytic core of all EPKs have highly conserved structural motifs. The glycine rich loop (yellow) and the C-helix (gold) from the N-lobe and the activation loop (red and green), the catalytic loop (orange), and the αF-helix (olive) from the C-lobe. ATP binds between the N- and C-lobes and the substrate binds to the C-lobe.

Phospho-Transfer

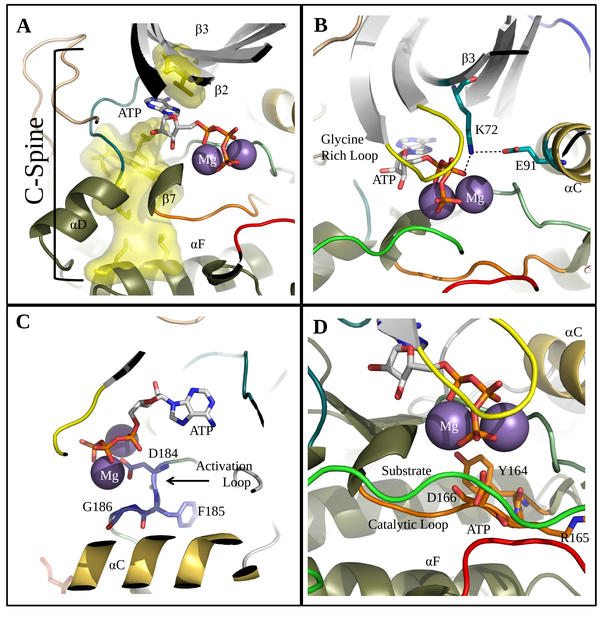

Figure 2: Phospho-Transfer in EPKs using PKA as a model. (A) The adenine ring of ATP completes the catalytic spine as it fits into the activation cleft. **(B)** The glycine rich loop (yellow) positions ATP and E91 (cyan) forms a salt bridge with K72 (cyan) that hydrolyzes the γ–phosphate. **(C)** The γ–phosphate is transported through the magnesium ions, that are positioned by ATP and D184 (blue) form the activation loop (DFG motif). **(D)** Substrate (green) is positioned to receive the γ–phosphate by the catalytic loop (HRD motif) (orange).

Note: Amino acid numbering is specific to PKA.

What allows this Dynamic Nature?

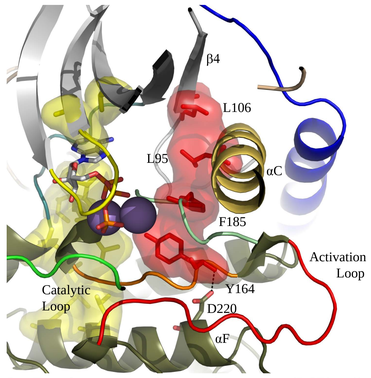

Figure 3: R-spine - Local spatial pattern (LSP) alignment allowed the detection of an aligned hydrophobic spine in active EPKs [5] which has provided a widely accepted framework for the mechanism [6] of EPK activity. These four residues include L106 from β4, L95 from the αC helix, F185 from the DFG motif from the activation loop, and Y164 from the H/YRD motif from the catalytic loop.

Non-Canonical R-Spine

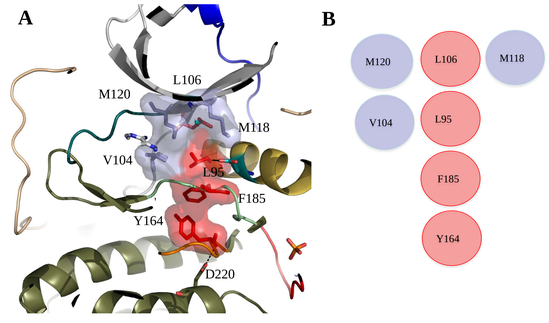

Figure 4: The R-spine was known to consist of only four residues. But, further computational analysis (LSP) has shown that there are other hydrophobic residues (M118, M120, V104). **(A)** Structural model of the non-canonical R-spine. **(**B) Cartoon model of non-canonical spine.

Preliminary Data

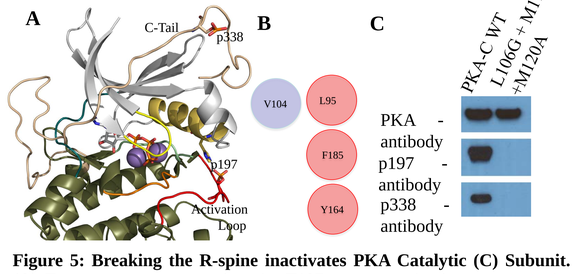

Figure 5: Breaking the R-spine inactivates PKA Catalytic (C) Subunit. (A) Trans-auto phosphorylation site T197 on the activation loop and cis-autophosphorylation site S338 on the C-tail. **(B)** R-spine broken by mutating L106G, M118A, and M120A. **(C)** Western Blot of wild type PKA-C and broken R-spine construct on both auto phosphorylation sites.

Role of the Gatekeeper

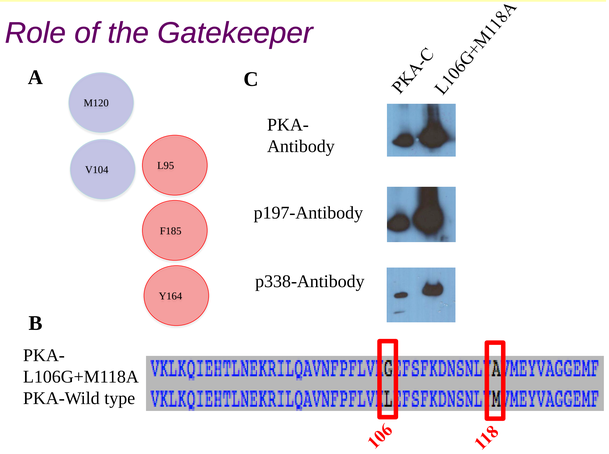

Figure 6: PKA-C R-spine completed by gatekeeper residue (M120). (A) Cartoon representation of gatekeeper R-spine. **(B)** Sequence of double mutant L106G and M118A in comparison to wild type PKA-C. **(C)** Western blot showing restored kinase activity of PKA-C double mutant on both auto phosphorylation sites.

Expression & Purification of Gatekeeper R-Spine

A

Quick change Mutation L106G+M118A	→	Transform into BL21 cells, (Overnight)	→	Transfer into 12 L flasks of LB media, 3-5 hours
OD~0.8 Induction, (Overnight)	→	Harvest & Resuspend	→	Lyse (Microfluidizer)
Centrifuge (17K, 1 hour)	→	Supernatant	→	Nickel Affinity Column
Wash x2 (10, 50 mM)	→	Elute (250 mM)	→	Dialysis

- **Buffer:** 50 mM KH_2PO_4, 20 mM Tris-HCl, 100 mM NaCl, 5 mM B-mercaptoethanol, pH 8.0
- **Dialysis Buffer:** 20 mM KH_2PO_4, 20 mM KCl, 25 mM DTT, pH 6.5

B

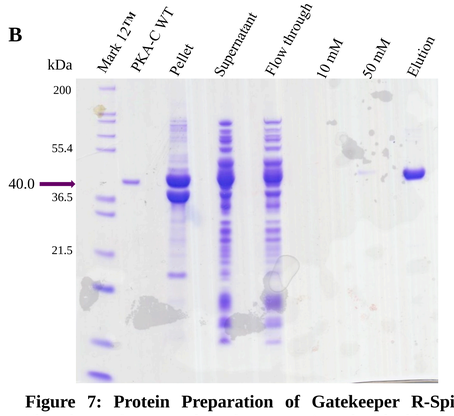

Figure 7: Protein Preparation of Gatekeeper R-Spine. (A) Flowchart of steps taken from creating the double mutant to purification of this construct. **(B)** Coomassie stained gel taken during different stages of the protein purification process of PKA-C double mutant.

Conclusions

- The gatekeeper residue (M120) was able to restore kinase activity (Figure 6C).
- Part of the expressed PKA-C double mutant went into the insoluble fraction (pellet (Figure 7B)).
- Some of the expressed PKA-C double mutant was lost during the loading process onto the Ni-NTA affinity column (flow through (Figure 7B)).
- None of the expressed PKA-C double mutant was lost during the 10 mM and 50 mM imidazole washes.
- A significantly pure PKA-C double mutant was eluted from the Ni-NTA affinity column (elution (Figure 7B)).
- The entire PKA-C double mutant elution precipitated during dialysis.

Future Directions

1. **Do L106 and M118 have the capability to restore kinase activity?**
   - To answer this question, the same experimental procedures will be carried out with the different constructs shown in Figure 8.
2. **Is the alignment of the R-spine restored?**
   - To answer this question. X-ray crystallography will be utilized to solve the structure of all three mutants (Figure 6A, 8A & 8B).
3. **Can rearrangement of these residues be utilized to design better drugs???**

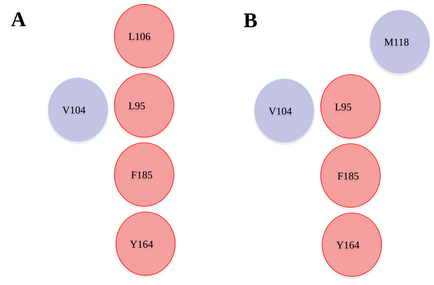

Figure8: Alternate conformations to the R-spine to test in future. (A) Cartoon model of L106 R-spine. **(B)** Cartoon model of M118 R-spine.

References

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