Sequence analysis of the PAK1 binding site for DGKζ

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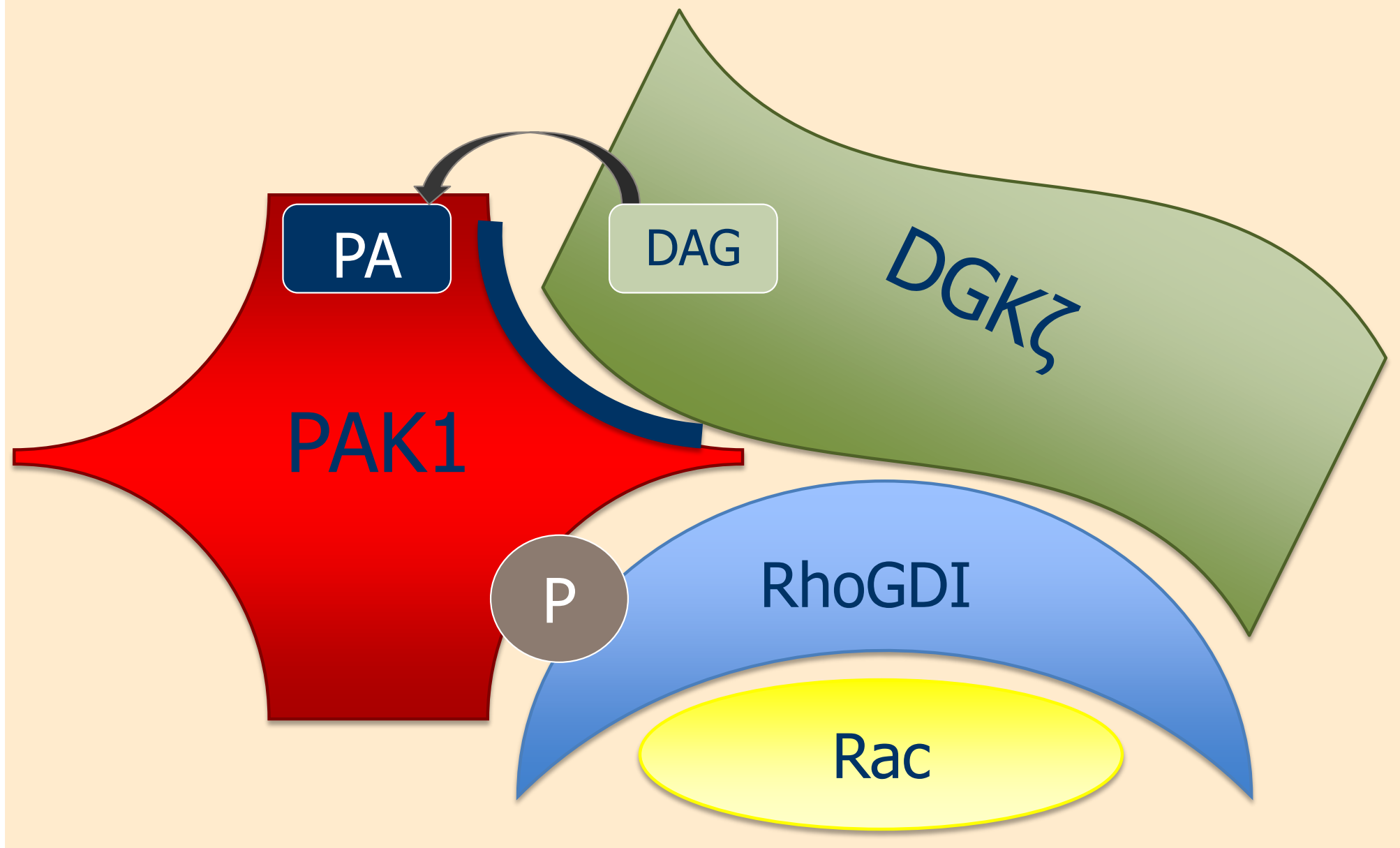
Abstract

Rho GTPases are key regulators of actin cytoskeleton organization. Deregulation of Rho GTPase function contributes to the cancer cell metastasis; therefore it is necessary that their activities are precisely controlled. Activation of the Rho GTPase Rac1 occurs by phosphorylation and dissociation of its inhibitor, RhoGDI, by the kinase PAK1. PAK1 is activated by phosphatidic acid, a membrane lipid produced by diacylglycerol kinase ζ (DGKζ)-mediated phosphorylation of diacylglycerol . A previous study by Abramovici *et al.* showed DGKζ directly binds PAK1 and RhoGDI to selectively dissociate Rac1. The binding site in PAK1 for DGKζ is contained within amino acids 35-56. To further characterize the interaction domain, PAK1 constructs with neutral amino acid substitutions in two potential binding sites, proline- and lysine-rich domains, respectively, were created and tested for their ability to capture DGKζ from mammalian cell extracts. No binding was observed to the mutated proline sequence, whereas attenuated binding was observed to the mutated lysine-rich region, suggesting the importance of both domains to DGKζ binding. Studies such as these will help elucidate the mechanism of actin cytoskeleton regulation and may identify new therapeutic targets for treating metastatic cancer.

Introduction

Amongst its many roles, Rac is an important regulator of actin cytoskeleton organization. When in its active state, RhoGDI binds and inhibits Rac function. RhoGDI inactivation is mediated by the kinase activity of PAK1. Phosphatidic acid (PA), produced by phosphorylation of diacylglycerol (DAG) by DGKζ, is an activator of PAK1.



Recent studies have also shown that DGKζ and PAK1 also **directly** bind to each other at amino acids 35-56 in PAK1. The purpose of this study is to narrow down the amino acids in PAK1 that are essential in its binding site for DGKζ. It is important to understand the interaction between the proteins of this pathway because improper actin cytoskeleton reorganization can lead to metastasis of cancer cells.

Methods

- Isolated various constructs of PAK1
- Performed a pull-down with DGKζ
- Observed results by Western Blotting

Six PAK1 constructs were obtained by induction of bacterial cultures containing plasmids coding for the proteins' genes:

361 : PAK1 Wild-Type
371 : PAK1 a.a. 35-56 only (binding site)
372 : PAK1 with a.a. 35-56 removed
433 : PAK1 a.a. 35-56 only; **P4→A**
434 : PAK1 a.a. 35-56 only; **K4→N**
435 : PAK1 constitutively active; a.a. 35-56 removed

The constructs were purified onto GST beads and their concentrations were determined by Bradford microassay.

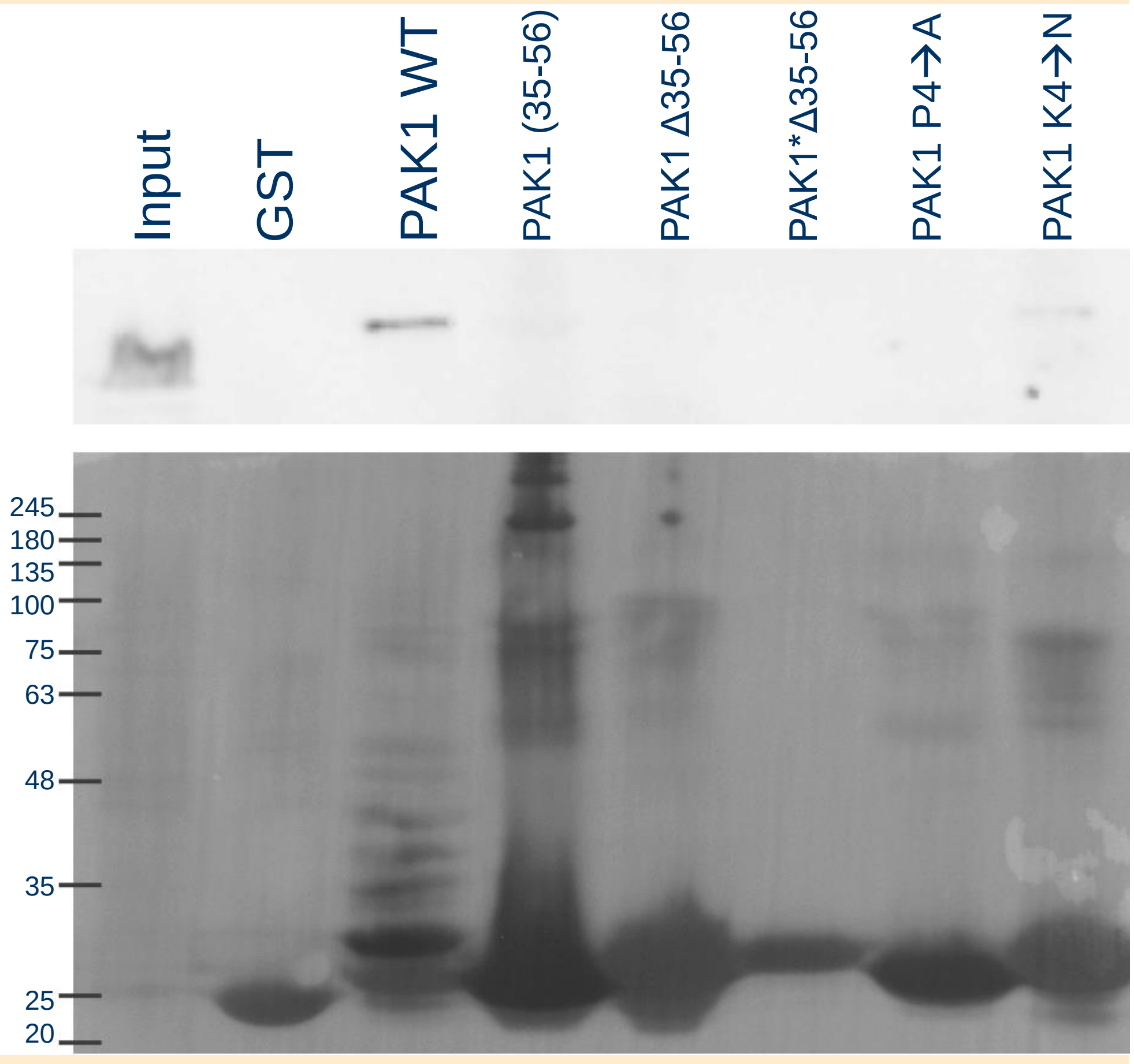
DGKζ was obtained by viral infection of the corresponding DNA into mouse embryonic fibroblast cells. Cell lysates containing DGKζ were added to solutions of each of the constructs and a *Glutathione Sepharose Transferase Co-Immunoprecipitation Pulldown* was performed. The interacting proteins were run on SDS-PAGE gel electrophoresis and stained with a Ponceau stain.

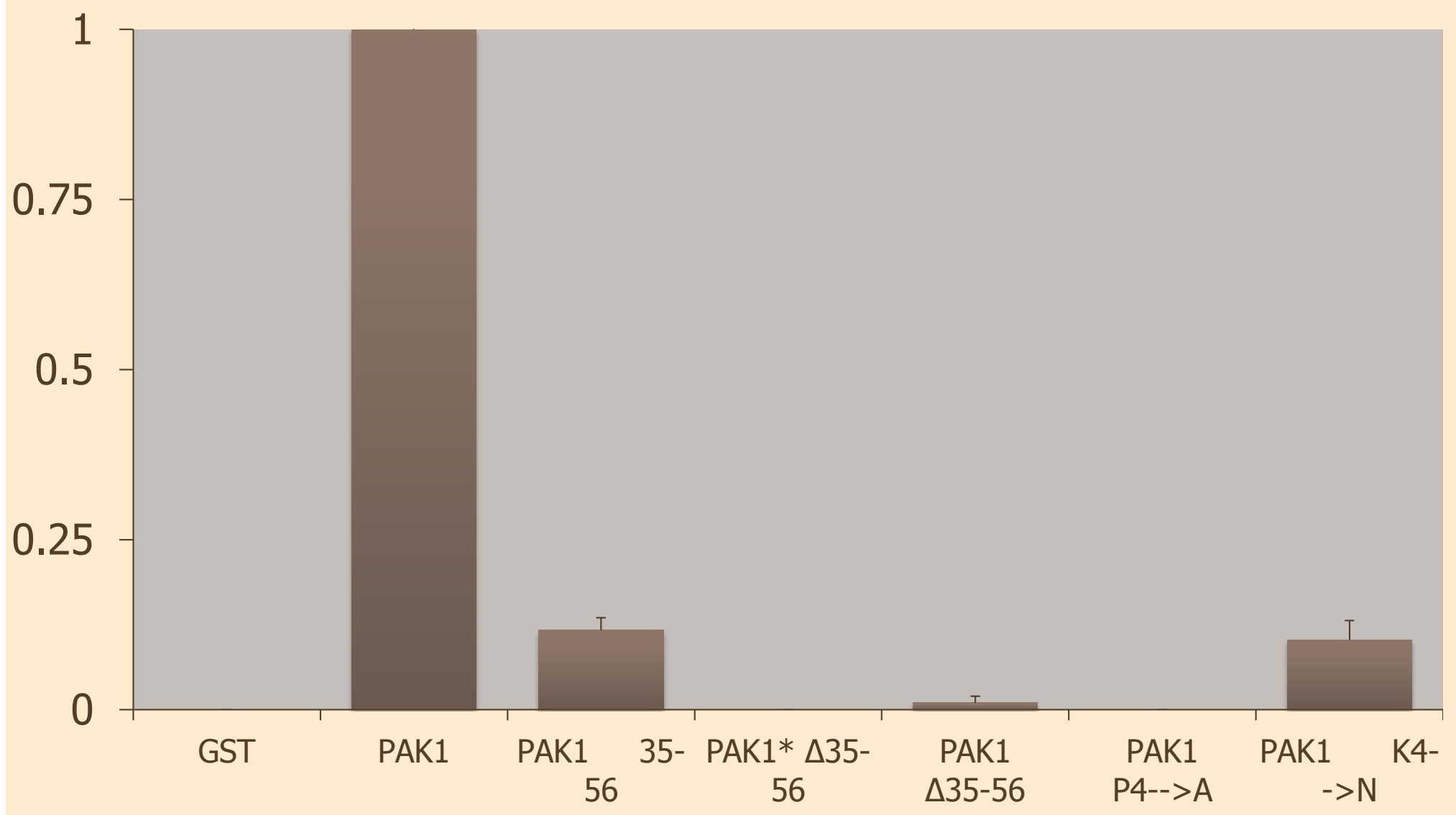
A Western Blot was used to quantify protein interaction. First, a mouse anti-HA antibody was used to detect presence of HA-tagged DGKζ. Donkey anti-mouse antibodies were used secondarily. Therefore, only those PAK1 constructs on GST beads which were bound to DGKζ appeared after light exposure.

N = 3 trials of this experiment were conducted.

Results

Western Blot





<u>0% binding</u> was shown between construct 433 (P4→A) and DGKζ.

<u>10.3% binding</u> was shown between construct 434 (K4→N) and DGKζ.

0% binding was shown between construct 435 (constitutively active with binding site removed) and DGKζ.

As expected, the Western Blot showed binding between DGKζ and constructs 361 and 371 (positive controls), and <u>no</u> significant binding between DGKζ and constructs 004 (GST beads only) and 372 (negative controls) for all three trials of the experiment.

Conclusion

The results of this study suggest the importance of the proline and lysine domains in the binding site of PAK1 for DGKζ. Specifically, when the proline residues are replaced with alanine, no binding with DGKζ is observed. When positively charged lysine residues are replaced with neutral asparagine, slightly attenuated binding can be observed, compared to the control. Further studies would be needed to concretely establish the role of the lysine residues in the PAK1 binding site for DGKζ.

Also, because constitutively active PAK1 with its binding site removed also did not bind to DGKζ, it as appears as though the activation of PAK1 does not modify its binding site for DGKζ.

Acknowledgements

Thank you to Dr. Stephen Gee for taking me on as a UROP student and allowing me to perform the experiments in his laboratory.

Thank you to Tanya Foley, Helga Agah and Dr. Kirk Mulatz for their indispensible help and guidance through my first laboratory experiments.

Schematic of DGKζ binding site on PAK1