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Identification of Ezrin as a Colonic Substrate for Protein Tyrosine Phosphatase Sigma

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Abstract – Protein tyrosine phosphatase sigma (PTPσ) is a transmembrane receptor-type protein tyrosine phosphatase. This enzyme contains several extracellular domains, a hydrophobic transmembrane stretch, and two intracellular phosphatase domains, D1 and D2, of which the D1 domain is catalytically active. PTPσ is encoded by the protein tyrosine phosphatase receptor S (Ptprs) gene. Previous research using single nucleotide polymorphism (SNP) analysis demonstrated PTPσ's association with inflammatory bowel disease (IBD). Ptprs-knockout mice spontaneously exhibit symptoms similar to human IBD. Although the genetic association of PTPRS and IBD has been identified, the role of PTPσ in the development of IBD is unknown. As the first step in understanding the role of PTPσ in IBD, it was critical to identify colonic PTPσ substrates. A list of putative substrates was previously generated using a phospho-tyrosine screen on PTPσ-knockout mouse colon tissue and served as a guide for experimentation. In this research, a substrate trapping assay was performed, revealing that the cytoskeletal linker protein, ezrin, was able to bind to the D1 domain of PTPσ in vitro. Furthermore, a phosphatase activity assay and an in vitro dephosphorylation assay confirmed the catalytically active D1 domain can directly dephosphorylate ezrin in vitro. These results strongly suggest that ezrin is a colonic substrate for PTPσ. Ezrin plays an essential role in organizing proteins at the apical membrane and prior studies have indicated ezrin is related to epithelial barrier defence, which could be associated with IBD. Characterization of colonic PTPσ substrates will greatly aid in understanding connections between PTPσ and IBD.

Key Terms - tyrosine phosphorylation, dephosphorylation, signal transduction, protein binding, colonic substrate, inflammatory bowel disease, knockout, wild-type, immunohistochemistry

1. INTRODUCTION

The Inflammatory Bowel Diseases (IBD) are chronic disorders of the gastrointestinal tract affecting over 200,000 individuals in Canada. Canada has one of the highest incidences of IBD in the world with 16.3 diagnoses per 100,000 for Crohn's disease and 12.9 diagnoses per 100,000 for ulcerative colitis[1]. Despite extensive research, the pathogenesis of IBD remains largely unknown. Previously, the Muise lab raised knockout mice for the receptor protein tyrosine phosphatase sigma (PTPσ). Interestingly, these mice developed IBD-like symptoms. Single nucleotide polymorphism analysis determined that PTPσ is genetically linked to IBD[2].

Tyrosine phosphorylation regulates many critical physiological functions. It is controlled by the balanced interactions between protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). PTPσ is a classical transmembrane receptor PTP. Its structure is outlined in Figure 1. Notably, the D1 phosphatase domain is the catalytically active site.

To better understand the role that PTPσ plays in the development of IBD, the first step is to identify its substrates in the colon. Colon samples from both wild-type (PTPσ+/+) and PTPσ knockout (PTPσ -/-) mice were screened for phospho-tyrosine containing residues and then run through tandem mass spectrometry to identify hyper-tyrosine phosphorylated proteins[3]. By comparing wild-type to knockout samples, a list of putative substrates was formed. The cytoskeletal scaffold protein ezrin was identified as a potential target.

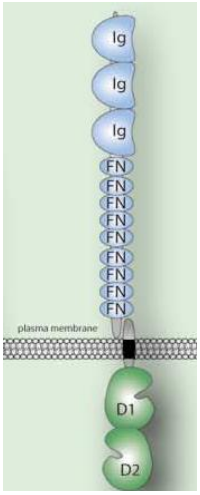

Figure 1: Domain Architecture of PTPσ

RPTPδ

2. PURPOSE

The purpose of this project is to identify, validate, and characterize the colonic substrates of PTPσ. Specifically; this work seeks to validate the interaction of PTPσ with the membrane protein ezrin.

3. HYPOTHESIS

Ezrin is a colonic substrate of PTPσ. PTPσ regulates the trafficking of ezrin to the apical membrane in enterocytes.

4. MATERIALS AND METHODS

4.1. *In vitro* Substrate-Trapping Assay

Colon samples from PTPσ-/- mice were homogenized using a tissue grinder and filtered using a 0.45μM filter. GST-fusion constructs for the D1 catalytic domain of PTPσ (GST-PTPσ-D1 (WT)) and D1 (DA) mutant (GST-PTPσ-D1 (D1472A)) were cultured in DH5α E. coli with IPTG stimulation. The harvested GST fusion proteins were incubated and bound to glutathione-agarose. The cleared colon lysates were incubated for one hour with GST-PTPσ -D1 (WT), GST-PTPσ-D1 (D1472A), and GST-only fusion proteins bound to glutathione-agarose. Following incubation, the beads were each washed twice with lysis buffer and low-salt HNTG buffer. Sample buffer was added to the beads which were boiled, and then run on a SDS-PAGE gel for Western blotting. A primary antibody to ezrin (mouse monoclonal) (BD Biosciences) and a secondary goat anti-mouse HRP conjugate (Jackson) were used for ECL-based visualisation.

4.2. Preparation of Constructs

N-terminal tagged FLAG ezrin constructs were generated from cDNA (SIDNET, Hospital for Sick Children, Toronto, CA) using the Gateway cloning system (Invitrogen) into a pcDNA 3.1-based human expression vector.

4.3. *In vitro* Dephosphorylation Assay

HEK293T cells were grown to 80% confluence on 100mm tissue cultures plates. 10mg of the pcDNA3.1 N-terminal FLAG ezrin plasmid was transiently transfected using the calcium precipitation method. Two days post-transfection, the cells were harvested in lysis buffer following a 15 min treatment with 50mM pervanadate. The ezrin protein was further purified through immunoprecipitation using FLAG-agarose (Sigma) followed by elution by 0.1M glycine pH 3.0. Bacterial cultures for GST-PTPσ-D1, GST-PTPσ-D1D2, GST-PTPσ-D2, and GST-PTPσ-D1 (D1472A) were grown, induced by IPTG, and harvested. The GST-fusion proteins were purified using glutathione-agarose and eluted with reduced glutathione. The catalytic activity of the GST-fusion constructs was tested using incubation with para-nitrophenyl phosphate (pNPP). The purified FLAG-ezrin was incubated with the purified GST-fusion PTPσ domains in 50 mM Tris-HCl pH 8.0 at 37°C. After 30 minutes, the reaction was stopped in sample buffer. Samples were run on a SDS-PAGE gel for Western blotting. Primary anti-FLAG (Sigma), anti-pTyr (4G10; Millipore), anti-GST (Covance), and anti-ezrin antibodies were used along with goat anti-mouse secondary antibody for ECL-based visualisation.

4.4. Immunohistochemistry

Paraffin-embedded, formalin-fixed PTPσ+/+ and PTPσ-/- mouse colonic tissue samples were sectioned into 5μM slices on a rotary microtome.

Sections were subjected to heat-induced epitope retrieval (HIER) in citrate buffer pH 6.0 for 20 min. Slides were deparaffinized in xylenes and re-hydrated. Blocking occurred for 1 hour in normal goat serum. Anti-ezrin primary antibody was incubated in a humidified chamber overnight at 4°C. Secondary goat anti-mouse HRP conjugate was incubated for 1 hour at room temperature. Staining was visualised using DAB (Sigma) for 5 min. Slides were counterstained with neutral blue, dehydrated back to xylenes, then mounted using Permount.

5. Results and Discussion

In order to verify that ezrin is a colonic substrate for PTPσ, an *in vitro* substrate-trapping assay was performed using the D1 domain and D1 (D1472A) mutant of PTPσ as baits. As seen in Figure 2-A, both the GST-PTPσ-D1 and GST-PTP-σ D1 (D1472A) mutant were able to precipitate endogenous mouse ezrin while the GST alone did not. This suggests an interaction between the D1 domain of PTPσ and ezrin. After verifying the catalytic activity of the GST-fusion constructs using pNPP absorbance at 405 nm (Figure 2-B), an *in vitro* dephosphorylation assay was performed using the FLAG-tagged ezrin as a substrate. For the incubations with the D1 and the D1D2 containing constructs, a marked decrease in total tyrosine phosphorylation is evident over the thirty minute incubation (Figure 2-C). This decrease was not seen in the case of the D2 domain or the catalytically dead D1 (D1472A) mutant, which is expected because the D1 domain is known to be catalytically active. Taken together, these results strongly suggest that PTPσ can dephosphorylate ezrin *in vitro*. Comparison of immunohistochemical staining for ezrin in PTPσ+/+ and PTPσ-/- mouse tissue (Figure 2-D) revealed no significant difference. However, further investigation will be necessary.

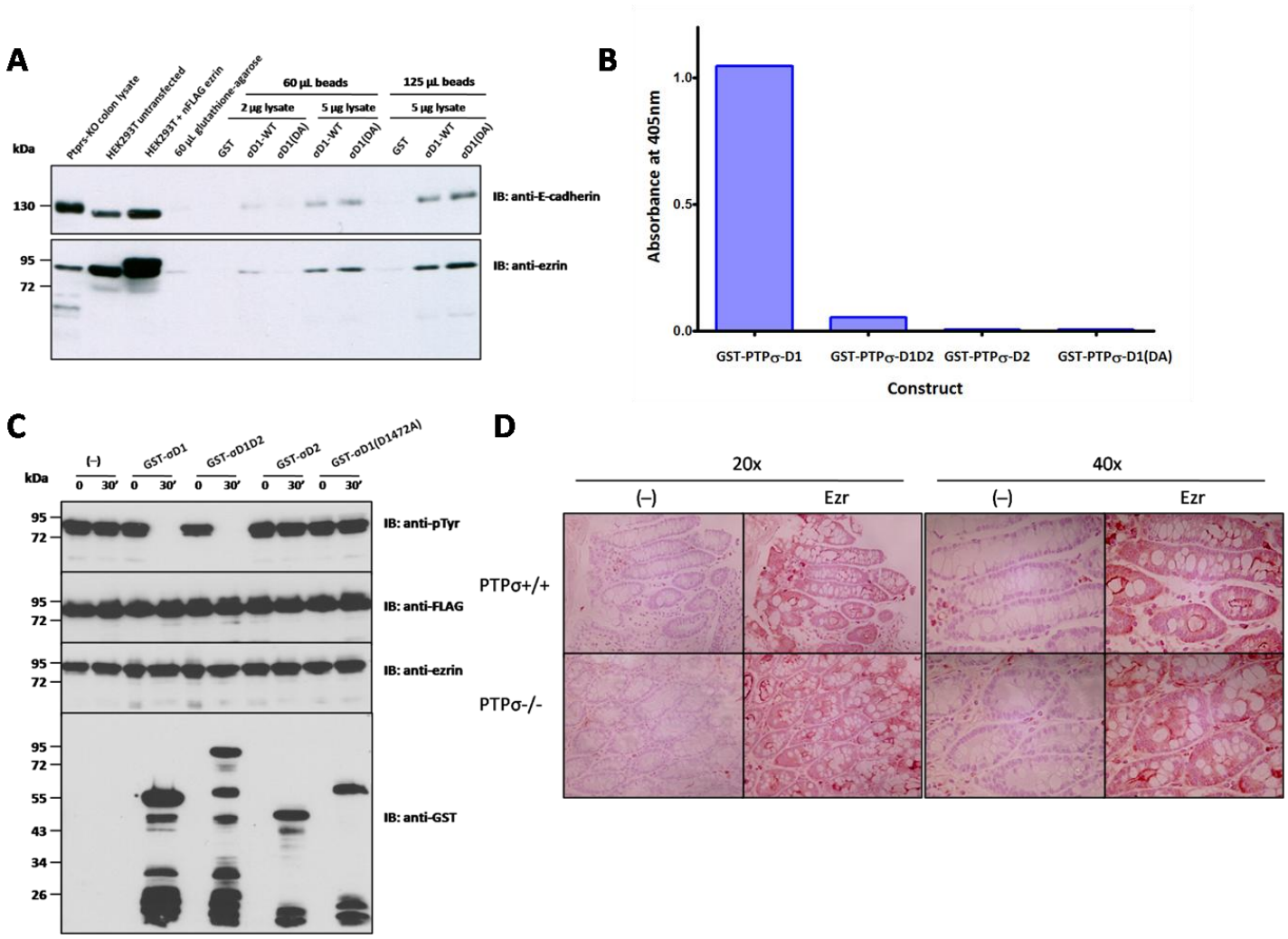

Figure 2: **A.** ***In Vitro Substrate-trapping assay using endogenous PTPσ-/- colonic tissue***. The D1 domain of PTPσ, conjugated to GST, was used as bait in a GST pulldown assay. A substrate trapping mutant (D1472A) of PTPσ was also used to increase captured protein. Various concentrations of lysate and GST-fusion protein were used. Detection of E-cadherin pull-down was used as a positive control. The GST only lanes were present as negative controls. **B.** ***pNPP assay for GST-fusion constructs***. GST fusion constructs for the intracellular domains of PTPσ were incubated with pNPP for thirty minutes. Absorbance values were read in a 96-well plate reader at room temperature. ***C. In vitro dephosphorylation assay***. Immunopurified, pervanadate treated ezrin was incubated with GST-fusion constructs representing the intracellular domains of PTPσ for thirty minutes at 37°C. A negative control with no GST-fusion protein added was included. ***D. Immunohistochemical staining for ezrin in PTP+/+ and PTP-/- mouse colon tissue.*** PTP+/+ and PTP-/- mouse colon tissue was sectioned and stained for ezrin. Two magnifications were used (20x and 40x). Negative control images stained with secondary antibody only are included. Nuclei were counter stained in blue.

6. CONCLUSIONS

Through the use of a substrate-trapping assay and colon samples from PTPσ-/- mice, ezrin was shown to bind and interact with the D1 domain of PTPσ *in vitro*. In addition, the catalytically active D1 domain of PTPσ dephosphorylated ezrin *in vitro*. These results taken together strongly suggest that ezrin is a colonic substrate for PTPσ. Further confirmation could be done by using *in vivo* techniques as well as co-localization studies. In the future, research will be conducted to identify the phosphorylation sites inside ezrin, to understand how PTPσ regulates ezrin, and, finally, to determine what role PTPσ and ezrin play in the pathogenesis of IBD.

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