The Significance of the Interaction between Plexin-B3 and Cdc42-GTP

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ABSTRACT

Axon extension in the nervous system during embryonic development is believed to be guided by numerous axon guidance molecules, which signal through their receptors on the surface of neuronal growth cone. One of the putative signaling mechanisms is the small Guanosine Triphosphatases (GTPases), and their downstream effector proteins that modulate actin cytoskeleton. Our laboratory has previously shown that the guidance molecule Plexin B3 interacts with the guanosine triphosphate (GTP) bound form of cell division cycle (Cdc42) plexin. This project aims to study how the presence of Plexin B3 could affect the availability of Cdc-42 GTP for its physiological effector WASP, thereby shedding light on the plausible axon guidance mechanism mediated by Plexin B3.

INTRODUCTION

Axons greatly rely on the actions of highly conserved families of attractive and repulsive cues such as Semaphorins and Ephrins for successful navigation towards the right targets. Semaphorins belong to a huge family of secreted and membrane–bound molecules which have a distinct \textit{sema} domain in them. In vivo, the interactions between Semaphorin ligands and Plexin receptors lead to an intracellular signaling cascade believed to be mediated by the highly conserved Sex Plexin (SP) domain found on the cytoplasmic region of Plexins \cite{1}. The presence of a consensus sequence in the cytoplasmic domain of Plexin, which share high homology with the Ras GTPase activating proteins (GAPs), may conceivably represent the link to translate extracellular guidance signals \cite{2}.

Plexins are a large family of receptors for transmembrane, secreted and the GPI-anchored Semaphorins in vertebrates \cite{3}. They can either act alone or in conjunction with neuropilins to initiate signal transduction pathway that controls cell repulsion. This signaling mechanism is crucial in axon guidance. In this study, we are interested in the significance of the interaction between Plexin-B3, a novel member of the Plexin family, and Cdc42-GTP. Previous findings in our laboratory have shown that the cytoplasmic domain of Plexin B3 interacts with the small GTPase, Cdc42. Interestingly this interaction maintains Cdc42 in the active GTP-bound state by slowing

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down the endogenous GTPase activity. Together with Rac1, Cdc42 has been known to be a positive regulator that promotes neurite extension, possibly acting through actin assembly that leads to the formation of lamellipodia and filopodia. Studies have proven that down regulation of small GTPases such as Rac1 and Cdc42 will lead to growth cone repulsion and collapse [4].

To date, Wiskott – Aldrich syndrome protein (WASP) and p21-activated kinase (PAK) are the two effector proteins downstream of Cdc42-GTP known to mediate various physiological functions. WASP interacts with Cdc42 and Rac via their Cdc42/Rac – interactive – binding (CRIB) motif [5]. It is postulated to link activated Cdc42 directly to the rearrangement of actin. Research has shown that the WASP GTPase Binding Domain (GBD), which includes the CRIB in the extended amino terminal, has a 500 – fold higher affinity for Cdc42 – GTP than Cdc42 – GDP [5]. Thus, the aim of our studies is to examine how the interaction between plexin-B3/Cdc42-GTP would affect the availability of Cdc42-GTP for its effectors. Here, we ask specifically if this interaction promotes the downstream signaling cascade of Cdc42 or does Plexin B3 compete with effectors like WASP to bind Cdc42 – GTP, thus turning off signaling cascades.

To understand the interactions, Maltose Binding Protein (MBP) fusion proteins with Cdc42 in the constitutive active (Q61L) and the dominant negative (T17N) forms have been synthesized for subsequent tests on its differential bindings to Plexin B3 and WASP. Due to time constraints however, my research has only progressed till the stage of protein expression of these two constructs.

Materials and Methods

PCR Amplification

Primers have been designed to incorporate EcoRI and XbaI restriction sites to flank the coding sequence of Cdc42 via Polymerase Chain Reaction. In the PCR reaction, Pfu Polymerase, 5pmoles of each of the forward and reverse primers, 200µM of dNTPs, and 0.5 µg of plasmids were used.

Mini Prep of Plasmid DNA

Miniprep of the plasmids was performed using the GFX™ Micro Plasmid Prep Kit from Pharmacia. This kit employs the usage of modified alkaline cell lysis procedures to isolate high yields of DNA. The solutions I, II, and III provided in the kit are used for the suspension, cell lysis, and neutralization of the culture respectively. The GFX columns and the wash buffer are used to isolate purified form of plasmids.

SDS PAGE

5µl of SDS PAGE sample buffer was added to 10µl of the sample and boiled at 95°C for 5 min. 15µl of each sample was loaded on each lane, and was run on a polyacrylamide gel (4%Stacking gel and 8% resolving gel, 1mm thick) at 200V. A protein marker (Pm) was run on the last lane. The gel obtained was stained using coomassie brilliant blue staining solution for an hour after which it was destained. The destained gel was dried using a vacuum pump (Fig 3).
RESULTS

The primer sequences that were used are as follows

5’ – CCGAATTCATGCAGACAATTAAGTGTGT – 3’ (mCdc42 Forward Primer)
5’ – CCTCTAGATTAGAATATACAGCACTTCC – 3’ (mCdc42 Reverse Primer)

Mus musculus Cdc42Q61L and Mus musculus Cdc42T17N constructs

The 580bp PCR amplified fragment of mCdc42Q61L and mCdc42T17N was subcloned into the EcoRV site of pCAP vector and used to transform DH5α E.coli strain. Transformants that survive on agar plates containing ampicillin were subjected to well lysis screening to identify the colonies that harbour the correct inserts. Based on the results, colonies Q1, Q3, and T1 were inoculated into 2ml LB broth (pH 7.5) containing 50µg/ml ampicillin and placed on a shaker at 220rpm overnight. Mini prep was performed to isolate purified plasmid DNA from the bacteria. Gel Electrophoresis was performed to reaffirm the colonies that contain the correct recombinant plasmids (Fig 1). The last lane shows the 1kb marker. Results show that Q1 and T1 contain the inserts. The size of the undigested pCAP vector (not shown in Fig 1) is slightly below 2kb. The size of the correct recombinant plasmids should be slightly above the 2kb mark as indicated by the bands marked with the arrow. This is because the size of the insert is 580bp.

Restriction Digestion to release inserts from pCAP recombinant plasmid

130ng of the recombinant plasmids was digested with EcoRI (20units/ml) and XbaI (20units/ml) restriction enzymes. They were then incubated at 37°C for 1.5hrs and isolated via gel electrophoresis using 1% agarose (Fig 2). The inserts were then excised and gel purified using the QIAquick Gel Extraction Kit. This kit has been designed for the extraction of DNA fragments (70bp – 10kb) from standard or low melt agarose in TAE (Tris-acetate/ EDTA) buffer. In this case, we use a 1% agarose gel. Small aliquots of the two constructs were digested to increase the efficiency of digestion. (Arrow is pointing towards the inserts (~580bp) released from the plasmids.)

Cloning into pMALC2 vector

These inserts were cloned into an EcoRI, XbaI digested pMALC2 prokaryotic protein expression vector containing the gene coding for Maltose Binding Protein. Before ligation the vector was dephosphorylated using Shrimp Alkaline Phosphatase to prevent self-ligation. The ligated product was transformed into DH5α E.coli strain. The colonies were screened by PCR screening to identify those that carry the correct plasmid. One colony was picked from each of the two transformants to isolate plasmid DNA for restriction analysis using EcoRI and XbaI. Gel electrophoresis of the samples confirmed the presence of the 580bp insert in recombinant pMALC2 (Fig 3).
Expression of the Recombinant Protein in BL21 strain of *E.coli*

For the expression of the recombinant protein, *BL21* strain of *E.coli* was transformed with the pMALC2-mCdc42Q61L (Q) and pMALC2-mCdc42T17N (T) and pMALC2 (P) (no insert) plasmids. Single colonies containing the construct were selected and inoculated into 2ml LB broth and grown overnight. 1ml of this overnight culture was inoculated into 2ml LB broth containing 50µg/ml ampicillin and 0.5mM of Isopropyl b-D-Thiogalactoside (IPTG) and incubated two hours at 37°C. The cells were then spun down and suspended in 300µl Column Buffer (20mM of Tris HCl (pH7.4), 200mM of NaCl and 1mM of EDTA) before being lysed by sonication. The lysate was purified using 100µl of Amylose Resin, which binds to MBP. After washing the bound resin was isolated and resuspended in 100µl PBS and finally separated from the fusion protein by boiling at 95°C for 5min in the presence of 1x SDS PAGE loading buffer. The eluate was then extracted after spinning down the resin and SDS PAGE was performed. The result (Fig 4) shows protein expression levels in uninduced lysate (1T, 1Q, 1P), induced lysate (2T, 2Q, 2P) and the eluate (3T, 3Q, 3P) containing the purified protein. In the uninduced state, the level of protein expression is relatively low and consistent for all the constructs. In the induced state, a significant increase can be seen in the expression level at certain regions indicated by the arrows. This shows that the induction of the MBP pTac promoter by IPTG has caused an up regulation in the fusion protein of interest. The expected size of the fusion protein is 58kDa.

**DISCUSSION**

The aim of our studies is to examine how the interaction between plexin-B3/Cdc42-GTP would affect the availability of Cdc42-GTP for its effectors. To understand the interactions, MBP fusion proteins with Cdc42 in the constitutive active (Q61L) and the dominant negative (T17N) forms have been synthesized for subsequent tests on its differential bindings to GST-Plexin B3 and GST-WASP fusion proteins. At the present stage of research, no conclusive results can be reached and only the MBP-Cdc42 fusion proteins in both forms have been produced. But based on similar studies that have been performed on Plexin B1, the small GTPase Rac, and p-21 Activated Kinase...
(PAK) it has been proven that Plexin B1/Rac interaction inhibits PAK activation. This means that Plexin B1 acts as a competitive inhibitor towards PAK. From these results, we can hypothesize that Plexin B3 may have a similar effect on WASP.

Other members of our laboratory have synthesized the GST fusion proteins, with WASP and the cytoplasmic domain of Plexin B3. To find the effects of the affinity of Cdc42 for WASP in the presence of varying concentrations of Plexin B3, Cdc42 pulldown assays will be performed. Here, it is essential to keep the concentrations of GST–WASP and MBP–Cdc42 constant throughout the experiments. In the control, GST–WASP and MBP–Cdc42 interaction will be measured using a Cdc42 pulldown assay. The proteins will be separated by Western Blot and accurately identified using antibodies directed against each of them. With the results of the control, an MBP-Cdc42 pull down assay will be performed in the presence of varying concentrations of GST-Plexin B3. The assay is then purified and the concentrations of the various proteins present in the pull down fraction are calculated. A lowering concentration of WASP in the pull down assay with increasing concentration of Plexin B3 may indicate competitive inhibition.

REFERENCES