Determination of the role of the N- and C-termini in localizing TCL/RhoJ to cellular membranes.

Brooke R. Tader

Biology and Chemistry Major

Advisor: Dr. Hamann

Bemidji State University
Honors Program
Department of Biology and Chemistry

Determination of the role of the N- and C- termini in localizing TCL/RhoJ to cellular membranes.

Brooke R Tader

Michael Hamann, Ph.D.
Professor of Biology

Date 4/11/2017

Season Ellison, Ph.D.
Assistant Professor of Interdisciplinary Studies
Director of Honors
and Liberal Education Programs

Date 4/10/2017

Mark Wallert, Ph.D.
Professor of Biology

Date 4/11/17

Table of Contents

Abstract

Rho GTPases are involved in several cellular processes, including cell motility and division. One specific Rho GTPase, TCL is an important factor in tumorigenesis in melanomas. It has been shown that when TCL is knocked out in mice, tumor growth has been shown to be reduced by 55% in lung carcinomas where melanoma cells have also been injected when compared to mice expressing wild type TCL. Although TCL's amino acid sequence is known, there is not much else known about TCL's mechanism of action or activation, meaning there is more research to be done on TCL. Experiments were conducted to determine how the N and C termini of TCL influences its localization, which can help in further elucidating protein functions and mechanisms of action. One experiment fused the first 24 and the last 21 amino acids of TCL to the fluorescent protein Venus. The second experiment used a YFP-tagged version of TCL, where one or both termini were deleted from the protein and/or the CAAX motif was mutated in the protein. The data shows the C-terminus to be enough to bring TCL to the membrane, while the role of the N-terminus in TCL localization is less clear. Without either terminus, TCL is found to be spread throughout the cytoplasm with no specific localization.

General Background

GTPases are proteins that are normally thought of as inactive when binding a molecule of GDP, are activated through the binding of GTP, and are involved in many biochemical signaling pathways.[1] GTPases are divided into two categories: small G-proteins and heterotrimeric G-proteins. Low molecular weight (or small) G-proteins are proteins made of one subunit that binds GTP and then activates or deactivates downstream effectors. There are about 150 low molecular weight GTPases, that are arranged into five families: Ras, Rho, Rab, Ran, and Arf.[2] One example of a LMW GTPase is Ras. Ras is activated through its guanine nucleotide exchange factor (GEF) Sos.[3] Sos helps Ras release the bound GDP and then bind a molecule of GTP. Once the GTP is bound, Ras can activate Raf, which starts a kinase cascade that can ultimately affect gene transcription leading to cell growth and division.[4] Heterotrimeric G-proteins are activated through a G-protein coupled receptor (GPCR) which catalyze a GDP-GTP exchange on the α subunit of the heterotrimeric G-protein.[5] The α subunit then separates from the β and γ subunits of the G-protein, leading to activation of downstream proteins. For the α subunit, GTP-loading and activation leads to the inhibition of adenylate cyclase, stopping the creation of cyclic AMP, while dissociation of the β/γ subunits allows them to bind and activate an ion channel, increasing the membrane's permeability to K^+ ions.[6]

Rho GTPases (Ras Homolog) are a family of about 20 low molecular weight GTPases shown to be involved in the cytoskeletal reorganization of cells, proliferation, angiogenesis, and tumorigenesis.[7–9] TCL is a Rho GTPase that is closely related to TC10. TCL is considered TC10 Like, giving it the name of TCL. TCL is also termed RhoJ for Ras homolog family designation J. TCL will be the designation used throughout this thesis. TCL and TC10 are both closely related to Cdc42 in terms of sequence homology and may have overlapping function. There have been

more experiments done to characterize Cdc42, and it has been found to have connections with TCL and another protein, Rac1, inside cells.[10] Blocking TCL's function has shown to help slow tumor growth and help to diminish tumor size.[8] A goal of the research presented in this thesis will be to determine the amino acid sequence of TCL that may cause TCL to have a greater effect on tumor growth and angiogenesis than TCL's closest relatives, TC10 and Cdc42. Under-expressing TCL made for smaller tumors. Over-expression of TCL allowed for larger tumors.[8]

Introduction

Angiogenesis is the formation of new blood vessels by extension and growth of endothelial cells lining blood vessels[11] while tumor angiogenesis is the growth of new blood vessels needed for the tumor to grow and is signaled for by the cancer cells through the activation of angiogenic factors, such as VEGF, which is an important stimulator of angiogenesis.[11,12] Angiogenesis allows the tumor to gain the nutrients needed to keep growing, and if angiogenesis does not occur, the tumor can only grow to 1-2mm in size and will not metastasize into a secondary tumor.

TCL is effective in targeting tumor angiogenesis and knocking out TCL expression in endothelial cells has been shown to help slow tumor growth. This lessened tumor growth through the under-expression of TCL was found through breeding mice that knock out TCL and instead express GFP by exchanging TCL's exon 1 with GFP. The mice were then implanted with Lewis lung carcinoma and melanoma cells. After 7 days, TCL levels were tested in the TCL-KO mice; TCL was found in high levels in the tumors. This finding was due to endogenous TCL being found in the tumor/cancer cells. These mice with knock out TCL showed a slowdown in tumor growth and much less vascularization due to impaired angiogenesis. When compared to wild type mice, the TCL-KO mice showed a 55% reduction in tumor growth due to diminished

angiogenesis.[8] TCL has therefore been suggested be a potential target to inhibit angiogenesis that is specific to tumor angiogenesis that does not cause serious side effects. This blocking of TCL expression could be an important step in angiogenic inhibition as current drugs that target tumor related angiogenesis have negative side effects, such as hypertension and hemorrhaging.

There is still little known about the exact expression and function of TCL in tumor angiogenesis, but due to TCL being endothelial-expressed and activated through vascular endothelial growth factor (VEGF),[7] it is shown to help in the growth of endothelial cells, which could be how TCL aids angiogenesis. Also, it is shown that TCL regulates endothelial cell migration and tube formation, as well as modulates actinomycin contractility, which is another reason why TCL is thought to be involved in both angiogenesis and tumor angiogenesis. At the molecular level, It was found that downregulating TCL expression increased focal adhesions and stress fibers in migrating cells, which slows angiogenesis.[7]

Although TCL seems to be a logical target to inhibit tumor-associated angiogenesis, little is known about its overall biochemistry and function in cells. Previous data regarding TCL has shown a distinct membrane and vesicular cellular localization that may help uncover a role for TCL in angiogenesis. TCL has some similarities to N- and K-Ras proteins, which show differing biological outputs due to their relative occupancy in organelles and signaling microdomains.[13] Membrane binding and targeting motifs rely on interactions between where it is encoded within C-terminal hypervariable region (HVR) and the host membrane. Classic studies reveal Ras proteins rely on farnesylation and palmitoylation or a polybasic stretch of amino acids for proper and stable binding. The farnesylation occurs at Ras's CAAX motif, where C stands for a cysteine residue, A stands for an aliphatic residue, and then the X stands for any amino acid. The CAAX motif can be found on many proteins' C-terminal tail in which prenylation can occur by the

covalent linkage of the cysteine residue to an isoprenoid group via a transferase protein. TCL also contains this CAAX motif, indicating that TCL can be prenylated, allowing for interactions between TCL and the cellular membrane. The linker domain of palmitoylated Ras HVR's is both where the third motif is present, as well as being necessary for anchoring Ras to the plasma membrane. Alterations in localization can happen without changing the status of palmitoylation. The data in this article provided a mechanism for interactions between the HVR and the membrane, which helped to control the distribution of Ras throughout the cell. This control of localization is important for controlling the signaling between Ras and Ras's downstream effectors.

Purpose Statement

Previous studies in the lab at Bemidji State University show that the first twenty amino acids are critical in the localization of TCL within the cell [14]. Full-length TCL localizes to the plasma membrane, however, deleting the first 20 amino acids (TCLΔN), localizes the protein to vesicles within the cell. In particular, the N-terminal amino acids 17-20 were found to be essential for membrane localization and GTP-loading of the GTPase.

Experiments will investigate how the N- and C- termini independently regulate TCL localization apart from its GTPase activity by fusing the termini sequences to the fluorescent protein Venus. Three constructs were generated; one where the N-terminus of TCL was fused to the N-terminus of Venus, one with the C-terminus of TCL fused to the C-terminus of Venus, and one with both TCL termini fused to the corresponding termini of Venus. These constructs were then used to test the hypothesis that the N- and C- termini of TCL facilitate localization of TCL without the core GTPase domain by transiently transfecting HeLa cells with the plasmids and visualizing the cells using fluorescence microscopy.

A second localization experiment involved the deletion or mutation of one or both termini from the full-length protein. Four new constructs were made for this experiment. The first construct involved was the deletion of only the C-terminus (TCLΔC), a second construct deleted both termini (TCLΔNΔC), a third construct had a mutation in CAAX box on the C-terminus (TCLΔAAX), and the fourth one deleted the N-terminus and a mutation in the CAAX box (TCLΔNΔAAX). These constructs were made to see the localization of TCL without one or both termini to determine which termini has the greatest effect on TCL's localization. The mutation of the CAAX box was done as other protein with this CAAX box are typically prenylated. TCL is thought to have a geranylgeranyl isoprenoid attached, rather than a farnesyl group like Ras, due to similarities in TCL's amino acid sequence to other proteins that are geranylgeranylated. This prenylation is what anchors a protein to the cellular membrane. The ΔAAX mutations involved change the cysteine residue to a serine, which inhibits the ability to covalently bind to the isoprenoid group, and the AAX portion is deleted entirely. By inhibiting this potential prenylation in TCL, one can also inhibit TCL's ability to localize to the membrane, thus showing how the CAAX box/C-terminus is essential in localizing TCL to the cellular membrane.

The intent of these studies is to compare how different changes in the DNA of the TCL plasmid affect where TCL localizes in HeLa cells. The independent variable is defined by the TCL mutant being used to transfect the HeLa cells. In the first experiment, these are labeled Venus, Venus C-Terminus, Venus N-Terminus, Venus C and N-terminus, TCL, and TCLΔN (delta N, meaning a change in the N terminus of TCL, in which the first twenty amino acids are deleted from the amino acid chain). In the second experiment, the independent variables are TCL, TCLΔN, Venus, TCLΔC, TCLΔNΔC, TCLΔAAX, and TCLΔNΔAAX. The dependent

variable is defined as where the TCL localizes in the transfected HeLa cells. The localization can be cytoplasmic, vesicular, or membrane localized (**Figs. 1** and **2**).

Figure 1. TCL localization in HeLa cells. Example of membrane localization.

Figure 2 TCLdN localization in HeLa cells. Example of vesicular localization.

Methods

Expression Constructs:

TCL and TCLΔN were cloned into PCI2.YFP plasmids using HINDIII and NOTI restriction enzyme sites. The TCLΔC and TCLΔAAX mutants were made using standard PCR based techniques. The Venus constructs were made using the PCI2.Venus plasmid and the DNA sequences for the appropriate termini.

Cell Culture and Transfection:

HeLa cells were cultures using RPMI-1640 media containing 5% (v/v) fetal bovine serum and 5% (v/v) bovine calf serum. Cells had to be trypsinized and replated the day prior to transfecting. Sterile coverslips were placed in 35-mm culture plates prior to seeding 0.8×10^6 cells per plate, along with 1 mL of the supplemented RPMI-1640 media. Lipofectamine-3000

was used for all transfections (Invitrogen). The cells were then incubated for approximately 24 hours.

Fluorescence Microscopy:

Images of fixed cells were captured at the University of North Dakota (Grand Forks, ND) microscopy core facility using a Zeiss LSM 510 Meta microscope using a 100x oil Plan-Fluor objective with a 1.45 numerical aperture. Images were assembled using Zen 2012 blue edition software.[14]

For analysis, three coverslips were transfected for a particular plasmid/construct. The day after transfections, coverslips were mounted to slides using ProLong Gold anti-fade mounting reagent. The coverslips were randomized, given a number, and counts were done in a blind experiment. Coverslips were looked at under an Olympus BH2 fluorescence microscope utilizing a 40x objective. At least 100 cells were counted per coverslip to determine the localization of TCL in the HeLa cells. Cells with YFP-fluorescence associated with the membrane were counted as membrane, cells with fluorescence associated with intracellular vesicles were counted as vesicular, and cells with fluorescence not associated with a specific part of the cell were counted as cytoplasmic.

Statistical Analysis:

Data are represented on the graphs as means ± SD. T-tests were performed between some of the data sets to determine if the difference is significant.

Results and Discussion

Venus experiment:

The Venus experiment (**Fig. 3**) revealed that the C-terminus of TCL may be of more importance than originally thought from the previous study at BSU. TCL itself looks like **Fig. 1** and is considered membrane localized. TCLΔN looks spotted throughout the cell, like **Fig. 2**, which equates to a vesicular localization. Venus shows a localization that is even throughout the whole cell, which is called cytoplasmic localization. These three constructs were used as controls for the three kinds of localization. The three constructs in which part of TCL is fused to Venus are the experimental constructs. The construct with TCL's C-terminus fused to Venus showed membrane localization, whereas, the N-terminus apart from the core GTPase domain showed cytoplasmic localization, meaning there is no specific pattern of localization and TCL is found throughout the cell. The N- and C-termini of TCL fused to Venus showed a membrane localization, like the C-terminus attached to Venus, but the difference was not significant.

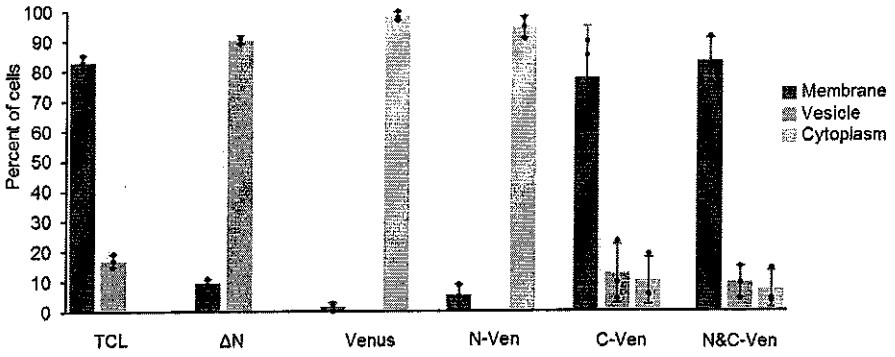

Figure 1. Graph of localization experiment involving Venus

Terminal Deletion Experiment:

The results of the experiment that deleted or mutated the termini of TCL showed the C-terminus is required in its non-mutated form for localization to the plasma membrane (**Fig. 4**). When the C-terminus was deleted or mutated from the TCL protein (ΔC or ΔAAX), most cells had localization to the cellular cytoplasm. When the N-terminus is deleted from TCL (ΔN, ΔNΔAAX, and ΔNΔC), there is some localization to the vesicles, but most cells still have TCL localizing to the cytoplasm. When the CAAX motif is mutated, there is some membrane localization, but most cells still showed a cytoplasmic localization. These findings are indicative of the necessity to have the full protein to localize TCL to the right place in the cell for proper function of TCL. Again, TCL, TCLΔN, and Venus were used as the localization controls for membrane, vesicular, and cytoplasmic, respectively. TCL and TCLΔN did not show the same significant localization to the membrane or vesicles, but from the previous experiment and the experiments done for the paper published last fall, these two constructs do show the membrane and vesicular localization necessary to be considered controls for this experiment.[14] Again, the differences in localization were not significant.

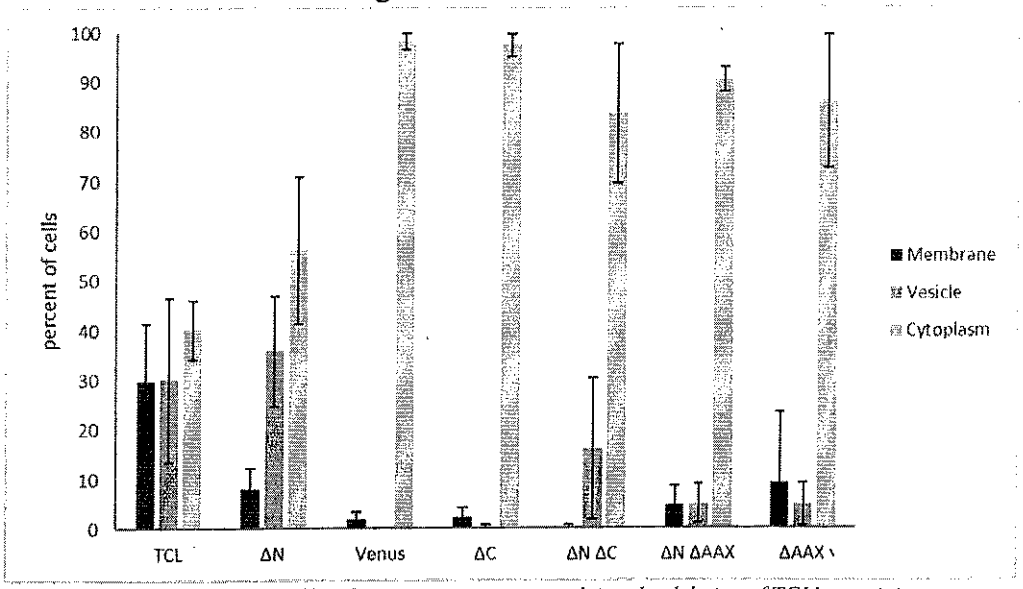

Figure 2. Graph of localization experiment involving the deletion of TCL's termini

Discussion/Conclusions:

When full length TCL is present, TCL abides in the membrane; deleting those first 20 amino acids makes TCL(ΔN) localize to the vesicles, but those same amino acids did not change localization when fused to a Venus protein. This could be due to the N-terminal amino acids interacting with the C-terminal amino acids to cause the membrane localization. Without the C-terminal amino acids present, the protein does not localize to the plasma membrane or vesicles, which furthers the idea that the C-terminus is most significant in localizing TCL to the plasma membrane, but the N-terminus is still necessary for membrane localization of TCL. These two pieces of information help to further the idea that the two termini interact to cause membrane localization.

What these experiments show is the C-terminus in its non-mutated form is necessary in the localization of TCL to the plasma membrane of the HeLa cells. The necessity of the C-terminus is seen when the localization is cytoplasmic when the C-terminus is deleted or the CAAX box is mutated. It is thought that active TCL is membrane bound, so this further leads to the thought that the C-terminus is needed for TCL to be active. The next experiments that could be done to test this idea would be to make constructs that are like the second localization experiment, except they would also contain a mutation inside the core domain of the protein that would make them constitutively active (always GTP bound) or dominant negative (always GDP bound). These constructs would contain either an T35N (dominant negative) mutation or a Q79L (constitutively active) mutation. This experimentation would be done to see how the activated (GTP bound) or inactivated (GDP bound) form of TCL with the terminal mutants would localize.

These findings are important in the continued efforts to find more targets in the fight against diseases like cancer. The more targets available to inhibit, the more personalized

chemotherapies can become. The research done here is only a small part of the larger picture of trying to find a treatment for diseases where TCL is involved, but it is still important as these details are necessary for further research into possible inhibitors. If normal TCL localizes to the membrane, then an inhibitor of TCL should be able to reach the membrane of a cell to work properly. This knowledge of localization can help in future research to find downstream effector protein of TCL, which could also become possible targets.

13. Laude AJ, Prior IA. Palmitoylation and localisation of RAS isoforms are modulated by the hypervariable linker domain. J Cell Sci. 2008;121(4):421–427.

14. Ackermann KL, Florke RR, Reyes SS, Tader BR, Hamann MJ. TCL/RhoJ Plasma Membrane Localization and Nucleotide Exchange Is Coordinately Regulated by Amino Acids within the N Terminus and a Distal Loop Region. Journal of Biological Chemistry. 2016;291(45):23604–23617.