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Determining the Effects of Palmitoylation on Rho Family GTPase TCL GTP-Binding Activity Using Palmitoyl Co-A

By

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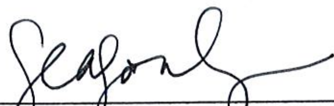

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Abstract

TCL/Rho J is a small GTPase involved in endothelial cell angiogenesis and is relatively abundant within some cancer cells such as melanoma and gastric cancers. This research is dedicated to understanding the biochemistry of this GTPase, and whether palmitoylation affects its activation and GTP-loading *in vitro*. To test palmitoylation, His-tag fusions of TCL, Cdc42, and Rac1 proteins were purified and treated with palmitoyl coenzyme A (palmitoyl-CoA), a compound that can palmitoylate protein cysteine residues *in vitro*. Palmitoyl-CoA treatment was found to decrease TCL's ability to load with the fluorescent nucleotide analogue MANT-GTP, measured using a GloMax fluorimeter. Interestingly, Cdc42 and Rac1 show more MANT-GTP loading activity, and were unaffected by treatment with palmitoyl-CoA. The data shows TCL GTP-loading activity may decrease when palmitoylated in cells, and may highlight a novel mechanism for regulating TCL.

Background

GTPases are hydrolytic proteins that are able to bind and hydrolyze guanine nucleotide triphosphate (GTP) within the G domain common to all GTPases. GTPases cycle between the active, GTP-bound, and inactive, guanine nucleotide diphosphate (GDP) bound form. GTPases act as regulators of various cellular functions that include cell proliferation, vesicular transport and membrane-trafficking through its on-off cycle.

Small GTPases are monomeric/single unit proteins that are 20-25 kD in size (Bos et al., 2007). Regulation of these GTPases are mediated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (Figure 1). GEFs catalyze the release of GDP to bind GTP and GAPs promote the hydrolytic activity of GTPases to remove the phosphate group on GTP (Bos et al., 2007).

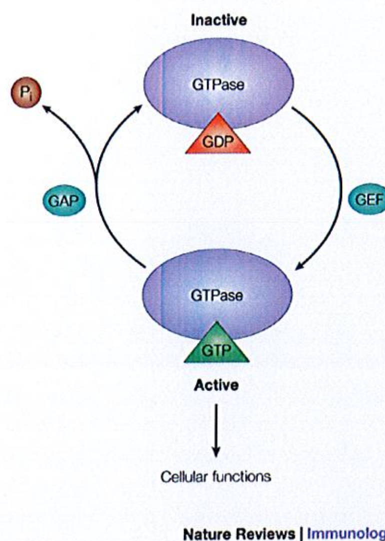

Figure 1. Diagram of GTPase nucleotide binding activity cycle, mediated by GAPs and GEFs. GTPases are activated by guanine nucleotide exchange factors by aiding in the release of GDP and loading of GTP. GTPases are inactivated by GTPase activating proteins, which promote the hydrolytic activity of GTPases to remove a phosphate group from GTP. Image is from (Taylor et al., 2004).

About 150 genes in the human genome encode small GTPases that are part of the Ras superfamily. The Ras superfamily is further subdivided into Ras, Rho, Ran, Arf, and Ran

families (Bos et al., 2007). The Rho family GTPases, including Rho, Rac1, and Cdc42 proteins, are involved in regulating cell shape, cytoskeleton dynamics, and cell migration (Bos et al., 2007). They have been shown to regulate the assembly and organization of filamentous actin in response to extracellular signals (Hall, 2012). Rho family GTPases also regulate extracellular matrix degradation, proliferation and capillary branching (Leszczynska et al., 2011). All Rho-family GTPases contain a Rho insert domain specific to this family (Wennerberg et al., 2004). The domain is found between the fifth β strand and fourth α helix within the GTPase domain. This Rho insert is important for downstream effector protein-protein interactions because it acts as a binding interface, but does not affect the stability or activity of GTPases (Vignal et al., 2000).

GTPases can undergo post-translational modifications that affect their activity within cells. Most Rho family GTPase are modified on their C-terminus though prenylation, which is the addition of a hydrophobic molecule including farnesyl and geranyl-geranyl isoprenoids to a C-terminal cysteine (Salaun et al., 2010). These additions assist with membrane association that are important to the function of GTPases (Roberts et al., 2008). Palmitoylation, another hydrophobic modification, is the reversible post-translational modification with 16 carbon-chain palmitate groups added to cysteine residues. In contrast to prenylation, which is the addition of a branched chain of carbons, palmitoylation is the addition of a long chain fatty acid. Palmitoylation can affect the activity of proteins which impact membrane interactions, intracellular sorting, stability and membrane micropatterning (Salaun et al., 2010). S-palmitoylation specifically refers to the addition of a palmitate group on cysteine residues through a thioester linkage (sulfur-sulfur bond) (Salaun et al., 2010). Palmitoylation is a highly regulated and coordinated system within cells (Salaun et al., 2010).

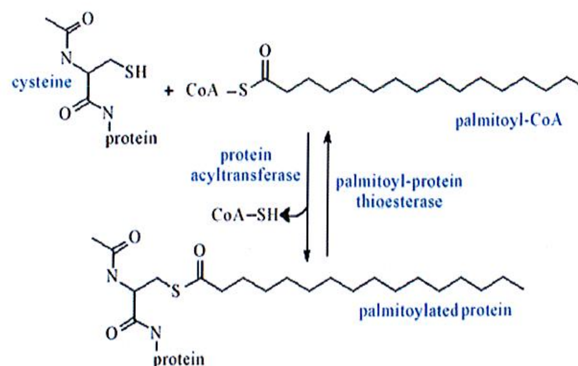

Figure 2. Protein S-palmitoylation – A palmitoyl group is being attatched to the cysteine protein by a thioester linkage, mitigated through protein acyltransferase, an enzyme that palmitoylates cysetin residues within cells.

Introduction

TCL/RhoJ, a Rho family GTPase, was characterized 16 years ago (Vignal et al., 2000). TCL is an abbreviation for TC10-like because TC10/RhoQ is closest in amino acid sequence similarity to TCL (Ackermann et al., 2016). TCL shares 85% of the same amino acids as TC10 with a similar N-terminal region and conserved effector loop, and also shares 78% of its amino acid sequence with Cdc42 (Vignal et al., 2000). Cdc42, TC10 and TCL may also share downstream effector proteins because they all bind to the Cdc42/Rac interaction binding (CRIB) domain of PAK and WASP (de Toledo et al., 2003; Vignal et al., 2000). These GTPases may share a domain that interacts with the CRIB domains of PAK and WASP specific to these proteins.

TCL is found primarily within endothelial cells, which are specialized cells that line blood and lymphatic vessels, unlike other family GTPases that are broadly expressed (Yuan et al., 2011). TCL is a target of ERG, a transcription factor that affects endothelial tube formation and angiogenesis within these cells, which is the formation of new blood vessels from older ones. (Yuan et al., 2011). Specifically, TCL has been demonstrated to regulate cell migration, tubulogenesis and lumen formation (Leszczynska et al., 2011).

TCL is found highly expressed in specific cancers including melanomas and some gastric cancers as well. Within melanomas, TCL regulates chemoresistance through the binding of downstream effector protein PAK1 that promotes a signal cascade resulting in the suppression of apoptosis in the presence of DNA damage along with changes in cytoskeleton dynamics (Ho et al., 2012, 2013). Overexpression of TCL in gastric cancer plays a role in increased motility and invasion in these cells as well making TCL a good target for chemotherapies (Kim et al., 2014, 2016).

Experiments done on mice in 2014 demonstrated the effectiveness of targeting TCL. TCL was found to promote tumor-associated angiogenesis in tumorigenic mice. The expression of TCL in mouse endothelial cells exhibited an increase of vascular growth in tumors. Deletion of TCL disrupted tumor vasculature integrity and function, reducing tumor growth within those cells (Kim et al., 2014). Targeting TCL by inhibiting its function within tumor cells could potentially stop angiogenesis and reduce tumor growth.

While disrupting TCL function in endothelial cells may be an important way to disrupt malignancies, it is also important to understand how TCL is regulated within cells. For GTPases, their cellular effects are often driven by localization within cells. A majority of Rho family GTPases undergo post-translational modifications that localizes the proteins to the plasma membrane or endomembranes needed for activity (Roberts et al., 2008). Rho family GTPases contain CAAX motifs, which contains three sites of posttranslational modifications needed for membrane association. The CAAX motif acts as a substrate for farnesyl or geranylgeranyl isoprenoid lipid additions. This CAAX motif is found on the C-terminus and contains a cysteine, C, an aliphatic amino acid, A, and a terminal amino acid, X (Roberts et. al., 2008). The CAAX motif however is not enough to fully associate the protein to the membrane nor can it fully target

the specific cellular subdomains. Two additional motifs upstream of the CAAX motif can be needed (Roberts et. al., 2008). One is a cluster of polybasic amino acids that provide the positive charge to facilitate the membrane associated lipids (Roberts et. al., 2008). Second are cysteine residues that undergo S-Palmitoylation, which can occur on some Rho family GTPases like RhoB and TC10 (Roberts et al., 2008).

Like its closest family member, TC10, TCL may also be palmitoylated. A specific region of TCL that encompasses amino acids 17-20 on the N-terminus were seen to be affecting the localization and nucleotide loading of TCL (Ackermann et al., 2016). Normally TCL is associated to the plasma membrane, but TCL becomes associated in intracellular vesicles when the N-terminus is removed (Ackermann et al., 2016). Furthermore, N-terminal deletions of TCL exhibit decreased nucleotide loading *in vitro*. However, when treated with 2-bromoplamitate, a palmitoylation inhibitor, cells expressing TCL localize to the vesicular membrane. TCL may need the post-translational modification to associate to the plasma membrane. Although experiments are ongoing in the lab regarding whether or not TCL is palmitoylated in cells, the experiments in this thesis sought to examine if direct, *in vitro* modification of TCL using palmitoyl-CoA could alter its GTP binding activity.

MANT-GTP loading assays can be used to determine the nucleotide binding activity of GTPases *in vitro*. MANT-GTP is a fluorescent nucleotide analog of GTP that contains the fluorophore, N-methyl-3'-O-anthranoyl (MANT), that fluoresces when binding to a GTPase (Remmers et al., 1994). The fluorescence can be measured and detected by an instrument to determine nucleotide-protein interactions. A Glomax fluorimeter is used for these experiments which outputs relative luminescence numerically on an excel sheet over time, and the nucleotide loading activity of GTPases can then be measured in this way by comparing the data points.

To obtain isolated protein for the assays, a technique called protein purification is used. A source, which could be cells from a culture or tissue from a plant or animal, is needed to produce the protein. The cell contents are then removed and the protein is isolated and purified by solubility, charge, size, or binding affinity (Berg et al., 2002). These experiments used affinity chromatography to isolate the proteins based upon the high affinity of His-fusion proteins to nickel resin. The His-fusion protein will remain on the resin while the rest of the cell contents are washed away, and then eluted with imidazole that release the protein from the resin to leave an isolated purified protein.

Methods

Cloning and Protein Purification

TCL GTP-loading activity was quantitatively measured *in vitro* using a MANT-GTP assay alongside Cdc42 and Rac1 to observe any distinct characteristics of palmitoylated TCL. His-fusion TCL, Cdc42 and Rac 1 proteins were expressed in *E. coli* strain bacterial cultures then purified in the absence of thiols (-SH group). Purification was performed using sonication lysis and His-nickel resin binding. A sample of the protein was loaded in an SDS-PAGE gel to determine the purity and concentration of purified product from BSA standards and gel densitometry.

Palmitoylation Reactions

Palmitoyl Co-A was used as the reagent to palmitoylate the proteins *in vitro*. His-constructs were used to reduce the amount of thiol groups that may be present in the buffer from the purification process. This prevented palmitoyl Co-A inactivation to keep palmitoyl Co-A from binding to thiol groups other than cysteine residues. The His-fusion proteins were diluted to 2 μM and treated with 25 μM palmitoyl-CoA.

MANT-GTP Loading Reactions

MANT-GTP loading assays were performed using 20 mM Tris, 50 mM NaCl, and 1 mM EDTA as a loading buffer containing 1 μM MANT-GTP. 1 μM fusion protein was then used, and the samples were read for 340 seconds at 10 second intervals. For the palmitoyl-CoA treated samples, 25 μM of palmitoyl Co-A was added the fusion protein. At 120 seconds $MgCl_2$ was added to stabilize TCL nucleotide binding. The samples were run in triplicate from the same initial isolation. Each assay was conducted with a negative control of glutathione s-transferase (GST), an untreated protein for a positive control, and palmitoyl Co-A treated sample. The data was normalized to make it comparable between the different data groups and graphed on a scatter plot to observe significant changes in GTP loading activity.

Results

The His- fusion proteins for TCL, Cdc42 and Rac1 were purified and loaded into a SDS-PAGE gel to determine the if the protein was purified and its appropriate molecular weight. The His-fusion proteins were relatively pure after the purification process seen by the distinct singular dark bands present on the gel (Figure 3). The relative concentrations of the proteins were calculated using gel densitometry so the same amounts of protein would be present in the MANT-GTP loading assays.

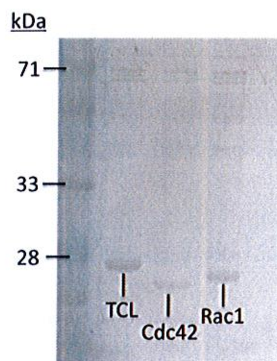

Figure 3. SDS-PAGE Gel of His-tagged TCL, Cdc42, and Rac 1. Nickle resin was used to isolate/purify the His constructs during purification. The protein concentrations were estimated using BSA standards and gel densitometry.

GTP-loading of TCL decreased when treated with palmitoyl-CoA (Figure 4). The TCL positive control showed a significant amount of loading in comparison to the negative control, GST, as seen by the error bars present in each data point. This difference displays that the GTP-loading was occurring in the purified proteins. Untreated TCL in comparison to palmitoyl-CoA treated TCL was a 200% increase in activity.

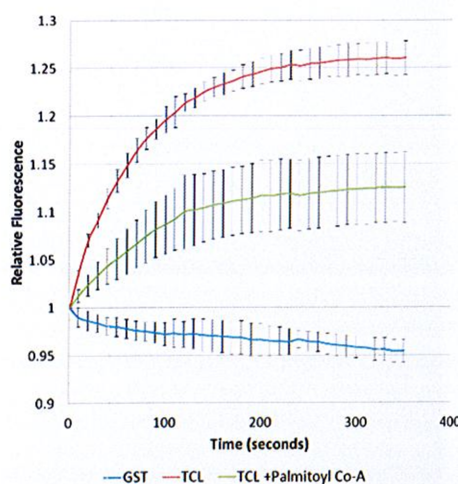

Figure 4. MANT-GTP loading assay of TCL indicates a decrease in GTP binding activity when Palmitoyl Co-A is added. Magnesium was added after 60 seconds of incubation. On graph, 1 second starts after the 60 second incubation period. 1 μM of TCL and 125 nM of MANT-GTP were used. 25 μM of palmitoyl Co-A was used in the +Palmitoyl Co-A. Average relative fluorescence and error bars were determined from triplicate samples.

However, Cdc42 and Rac 1 showed significant change of GTP-loading activity when treated with palmitoyl-CoA (Figure 5 and 6). The negative control, GST, exhibited no fluorescent activity in each of the assays as expected. The positive controls exhibited nucleotide loading significantly higher than the GST.

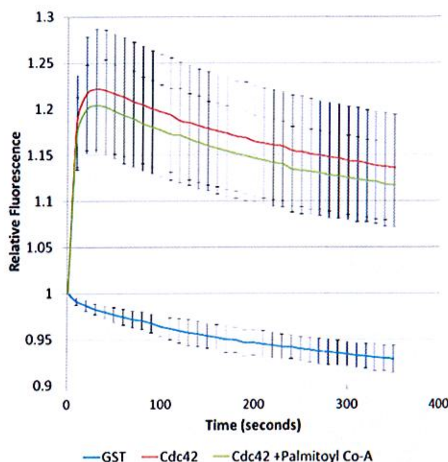

Figure 5. MANT-GTP loading assay of Cdc42 indicates little change in GTP binding activity when Palmitoyl Co-A is added. 1 μM of Cdc42 and 125 nM of MANT-GTP were used. 25 μM of palmitoyl Co-A was used in the +Palmitoyl Co-A. Average relative fluorescence and error bars were determined form triplicate samples.

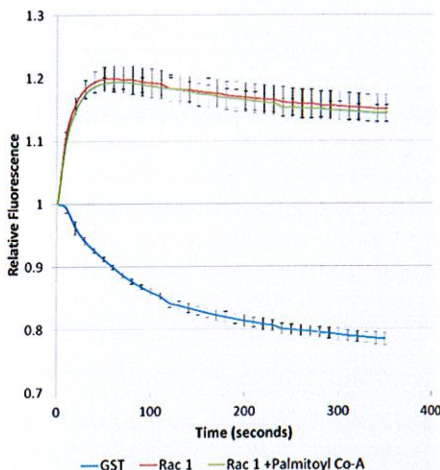

Figure 6. MANT-GTP loading assay of Rac 1 indicates little change in GTP binding activity when Palmitoyl Co-A is added. 1 μM of Rac 1 and 125 nM of MANT-GTP were used. 25 μM of palmitoyl Co-A was used in the +Palmitoyl Co-A. Average relative fluorescence and error bars were determined form triplicate samples.

Discussion

GTP-loading of TCL significantly decreased when treated with palmitoyl-CoA and Rac1 and Cdc42 did not have any significant change. These results may mean palmitoylation of TCL decreases its activity *in vitro* and that it has no effect on Rac1 and Cdc42 nucleotide loading activity. There is no way of knowing how palmitoyl-CoA interacts with the proteins in these assays. It is unknown whether palmitoyl-CoA is palmitoylating a cysteine residue or affecting TCL in an entirely different way. To determine whether palmitoyl-CoA palmitoylates TCL, an experimient that pin points where palmitoylation occurs would allow direct manipulation of that site for testing palmitoylation. TCL with a point mutation of the residue where palmitoylation occurs would exhibit TCL-nucleotide binding like that of non-palmitoylated TCL in the MANT-GTP assay if palmitoyl-CoA did palmitoylate TCL.

The nucleotide binding curves decreases over the course of the assay, and can be seen more prominently in the Cdc42 and Rac 1 graphs. The decrease of fluorescence over the period of time may be due to photo bleaching of the MANT fluorophore.

Conclusion

TCL GTP loading activity may decrease *in vitro* when palmitoylated and Cdc42 and Rac1 may not be palmitoylated or is unaffected by palmitoyl-CoA entirely. Due to the difference in GTP loading activity between Rac 1, Cdc42, and TCL, palmitoyl CoA is not arbitrarily affecting these GTPases, but rather targeting/affecting TCL specifically. However, there is not enough evidence to support that palmitoyl-CoA is palmitoylating TCL and further tests must be done to clarify how exactly palmitoyl-Co-A is affecting TCL nucleotide loading.

If these results are due to palmitoylation, it may prove to be another way TCL nucleotide loading activity is regulated within cells. These results give insight into a specialized difference

between TCL and some of its close relatives, which provides more information about TCL to create therapies that could reduce TCL's ability to promote cancer growth.

References

Ackermann, K.L., Florke, R.R., Reyes, S.S., Tader, B.R., and Hamann, M.J. (2016). TCL/RhoJ Plasma Membrane Localization and Nucleotide Exchange Is Coordinately Regulated by Amino Acids within the N Terminus and a Distal Loop Region. J. Biol. Chem. *291*, 23604–23617.

Berg, J.M., Tymoczko, J.L., and Stryer, L. (2002). The Purification of Proteins Is an Essential First Step in Understanding Their Function. Biochemistry, 5 ed. 4.1.1-4.1.7

Bos, J.L., Rehmann, H., and Wittinghofer, A. (2007). GEFs and GAPs: critical elements in the control of small G proteins. Cell *129*, 865–877.

Hall, A. (2012). Rho family GTPases. Biochem. Soc. Trans. *40*, 1378–1382.

Ho, H., Aruri, J., Kapadia, R., Mehr, H., White, M.A., and Ganesan, A.K. (2012). RhoJ Regulates Melanoma Chemoresistance by Suppressing Pathways That Sense DNA Damage. Cancer Res. *72*, 5516–5528.

Ho, H., Soto Hopkin, A., Kapadia, R., Vasudeva, P., Schilling, J., and Ganesan, A.K. (2013). RhoJ modulates melanoma invasion by altering actin cytoskeletal dynamics. Pigment Cell Melanoma Res. *26*, 218–225.

Kim, C., Yang, H., Fukushima, Y., Saw, P.E., Lee, J., Park, J.-S., Park, I., Jung, J., Kataoka, H., Lee, D., et al. (2014). Vascular RhoJ is an effective and selective target for tumor angiogenesis and vascular disruption. Cancer Cell *25*, 102–117.

Kim, C., Yang, H., Park, I., Chon, H.J., Kim, J.H., Kwon, W.S., Lee, W.S., Kim, T.S., and Rha, S.Y. (2016). Rho GTPase RhoJ is Associated with Gastric Cancer Progression and Metastasis. J. Cancer *7*, 1550–1556.

Leszczynska, K., Kaur, S., Wilson, E., Bicknell, R., and Heath, V.L. (2011). The role of RhoJ in endothelial cell biology and angiogenesis. Biochem. Soc. Trans. *39*, 1606–1611.

Remmers, A.E., Posner, R., and Neubig, R.R. (1994). Fluorescent guanine nucleotide analogs and G protein activation. J. Biol. Chem. *269*, 13771–13778.

Roberts, P. J., Mitin, N., Keller, P. J., Chenette E. J., Madigan, J. P., Currin, R.O., Cox, A. D., Wilson, O., Kirschmeier, P., Der, C. J. (2008) Rho Family GTPase Modification and Dependence on CAAX Motif-signaled Posttranslational Modification. JBC *283*, 25150-25163.

Salaun, C., Greaves, J., and Chamberlain, L.H. (2010). The intracellular dynamic of protein palmitoylation. J. Cell Biol. *191*, 1229–1238.

Shi, T., Li, G., and Xiao, H.-T. (2016). The Role of RhoJ in Endothelial Cell Biology and Tumor Pathology. BioMed Res. Int. *2016*, 638-641.

Taylor, G.A., Feng, C.G., and Sher, A. (2004). GTPases: regulators of immunity to intracellular pathogens. Nat. Rev. Immunol. *4*, 100–109.

Toledo, M., Senic-Matuglia, F., Salamero, J., Uze, G., Comunale, F., Fort, P., and Blangy, A. (2003). The GTP/GDP Cycling of Rho GTPase TCL Is an Essential Regulator of the Early Endocytic Pathway. Mol. Biol. Cell *14*, 4846–4856.

Vignal, E., De Toledo, M., Comunale, F., Ladopoulou, A., Gauthier-Rouvière, C., Blangy, A., and Fort, P. (2000). Characterization of TCL, a new GTPase of the rho family related to TC10 andCcdc42. J. Biol. Chem. *275*, 36457–36464

Wennerbert, K. and Der C. J. (2004) Rho-family GTPases: It's Not Only Rac and Rho. J. Cell Science *117*, 1301-1312.

Yuan, L., Sacharidou, A., Stratman, A.N., Le Bras, A., Zwiers, P.J., Spokes, K., Bhasin, M., Shih, S.-C., Nagy, J.A., Molema, G., et al. (2011). RhoJ is an endothelial cell-restricted Rho GTPase that mediates vascular morphogenesis and is regulated by the transcription factor ERG. Blood *118*, 1145–1153.