

Characterization of a Novel GEF KIAA1362

Erin Roach

Bemidji State University

Dr. Mike Hamann

Characterization of a novel GEF KIAA 1362

Introduction

The Rho-family of guanine triphosphate (GTP) binding proteins are important in intracellular communication of signaling pathways functioning as complex molecular switches for processes like cytoskeletal organization, cellular growth, migration, and cell fate (1-3). Activation of the GTPases is often induced at the cell surface by coupled variety of membrane bound receptors including G-protein coupled receptors. (4). There are about 20 mammalian members of the Rho GTPase family including Rho A, Rho D, Rho E, Rho F, Rho H, Chp, Miro, and Rac 1 that are responsible for the intracellular changes (5). Rho family proteins have been shown to lead to bundling of actin filaments, lamellipodia, and stress fibers (1) and through the use of these fibers, cellular migration is performed with the cooperation of contracting fibers and lamellipodia protrusions (3). Rho family members have been shown to activate transcription factors directly involved in cell cycle regulation and the apoptosis processes, which without proper functioning, can lead to continued cell growth and formation and eventually cancer (2).

The GTPases are activated by the conformational changes induced by the binding of GTP or guanine diphosphate (GDP) within the active site of the GTPase (6). While a GTPase is a GTP bound, it is considered to be in an active state and while a GDP is bound the protein is considered to be in an inactive state. These conformational changes follow a “lock and key” type mechanism where the nucleotide and an Mg^{+2} co-factor are slid into the rigid areas of the GTPase and is then bound there by conformational changes of switch regions around the active site (7).

The cycling between these GDP and GTP bound states is primarily the responsibility of two regulatory proteins, GTPase activating proteins (GAP) and guanine nucleotide exchange factors (GEF) (8). GAPs arrange the GTPase for hydrolysis of the GTP to a GDP form, sending the GTPase into an

inactive state. GTPases have a weak natural ability to hydrolyze the GTP, but GAPs greatly increase the rate of GTP hydrolysis. GEFs on the other hand, help to stimulate the activated state by stabilizing the intermediate to a guanine nucleotide free form, which in turn rapidly binds with GTP due to high intracellular concentrations. An important third, smaller family of proteins called guanine nucleotide-dissociation inhibitors (GDIs) also exists and their function is to lock the GTPase in a GDP bound state within the cytosol of the cell.

An important part of GEF stimulation is its ability to bind to GTPase through two consecutive structures called the Dbl homology (DH) domain and pleckstrin homology (PH) domain. These domains are present on all Rho-family GEFs and are key to GTPase interactions, leading to proper binding and improved catalytic function. The PH domain is thought to be responsible for cellular distribution or location, while the DH domain is mostly responsible for cellular communication (9).

Other important binding sites with the GTPase are the previously mentioned switches and a binding region known as the phosphate loop or p-loop. Switch 1 is part of the active site that is responsible for disrupting the binding of GDP and the Mg^{+2} upon GEF activation. Switch 2 has a very similar function but is located directly across the binding pocket of the GTPase from switch 1. The p-loop's purpose within the GTPase is to form a weak bond between the GTPase and the alpha and beta phosphate groups on the nucleotide base and changes only slightly when the phosphates are out of range (10). DH domains are highly conserved and interact extensively with the conserved regions of the GEF to assist with orientation about the GTPase.

Rho-family GEFs, GTPases, and their effector proteins have also been closely linked to many human ailments, including cancer and developmental or neurological disorders. For example, the deletion of the gene coding for GEF Fgd1 has been found in patients with faciogenital dysplasia (11). Faciogenital dysplasia disease is expressed as widely spaced eyes, front facing nostrils and some digital

malformation and overextension. Certain mental X-linked retardation has been shown to be influenced by Rho-family mutations (12). Rho-family GTPases are also major targets for viral and bacterial pathogenesis. Human immunodeficiency virus-1 (HIV-1) protein Nef binds to and activates Vav and is essential to the progression to AIDS (13).

By studying specific GEFs and GTPase interactions, it may be possible to target particular outcomes for therapeutic uses. The Rho-family is very diverse with 69 distinct GEFs and many still uncharacterized. With so many GEFs and only a small handful of GTPases, many redundancies within the family exist. These redundancies may be due to the specific location of certain GTPases to certain tissues in the body. With the localization of the GTPases, it follows that many of these GEFs may directly link with the cell's function within a specific tissue. The understanding of target tissues for these GTPases may lead to significant improvements in their use as a therapeutic target for certain ailments. If function, location, and triggers are identified for these GTPases there are many new levels of understanding and therapeutic hope for these proteins.

KIAA 1362 is a GEF within the Rho family that has had several of its closest relatives already characterized. Little is known outside of the actual amino acid sequence of the KIAA 1362 and inferred knowledge based on its close relatives. KIAA 1362 has been sequenced to be 1400 amino acids long consisting of two PH domains, a DH domain, a FYVE domain and a poly serine rich zone (14). The poly serine rich zone has yet to have any specific function in this protein identified, but it most likely functions as a phosphorylation site for cellular signaling. The FYVE domain is a specific type of zinc finger binding area shown to bind two Zn^{+2} ions. The FYVE domain is actually named after the first four proteins it was found in, Fab1, YOTB, Vac1, and EEA1. Function of the FYVE domain has been found to be membrane recruitment of the phosphatidylinositol 3-phosphate (PI3P) found commonly on endosomes (15). This current study focused on characterizing the GTPase that the GEF

KIAA 1362 would activate, which would help to define the GEF's purpose on a cellular and organismal level.

Methods

Primer DNA sequences flanking the DH and PH domains of GEF KIAA 1362 were synthesized by Integrated DNA Technologies. The primers were used for polymerase chain reaction (PCR) amplification along with a cDNA pool, and Phusion polymerase. The PCR product was purified from the unreacted reagents using a mini prep resin. The purified PCR product was then digested with the restriction enzymes HindIII and NotI enzymes. Samples of the PCR product were run to compare the DNA sample size to that of a 1 kb ladder standard in a 1% TAE agarose gel.

The product was then isolated from the gel by dissolving the gel containing the DNA fragment with sodium iodide, binding the DNA to ground glass (glass milk), and washing the glass milk with an ethanol-containing solution. The isolated DNA was then ligated into a pMAL plasmid. The pMAL plasmid contains a selective marker for antibiotic ampicillin within its construct.

The ligation reaction was used to transform DH5α *Escherichia coli*. The heat shock method utilized the permeability of the cell membrane of bacteria at the higher temperatures to transform the ligation mixture. After the transformation the bacteria were incubated at 37° C on a nutrient agar plates containing ampicillin overnight to selectively grow only those bacteria transformed with the pMAL plasmid.

After isolating the colonies that grew in the nutrient agar, a starter culture of Luria Bertani (LB) media containing ampicillin was inoculated for induction. The starter culture then inoculated a 100mL culture left to grow at 37° C until an OD₆₀₀ was reached. After the proper OD level was reached expression of the protein was induced using 100mM IPTG to create a final concentration of 100μM

IPTG and left overnight at 18° C . Purification of the stimulated protein was performed using the sonification technique. The sonication of the bacteria lysate solution helps to break open the bacterial cells without harming the protein. The lysate is suspended in a GSH agarose slurry before being washed with many solutions of a neutral wash with $MgCl_2$ solution. After isolating the protein from the waste products with the help of the resin. The protein is then assayed for concentration using a Bio-Rad Bradford Reagent.

An SDS-PAGE gel was used to check the purity and stained with Coomassie blue. The isolated GEF protein was then tested using a GTP/GDP fluorescent exchange assay with four major Rho-family GTPases, Cdc42, Rho A, Rac1, and Vav1.

Results

The figure below shows an agarose gel after having the PCR product checked for valid sizes. Lane 1 consists of the 1 kb ladder. Lane 2 consists of the GEF KIAA 1362. The DNA found in lane 2 is consistent with the size of the DNA researcher started the PCR with showing a positive result from the PCR.

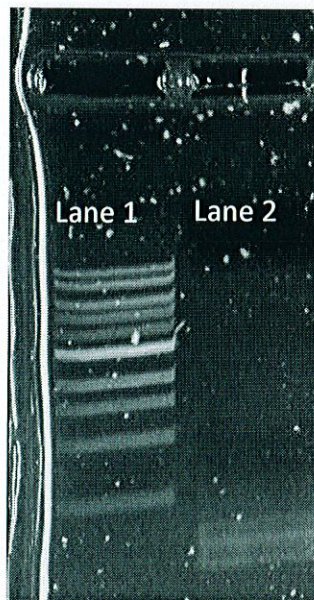
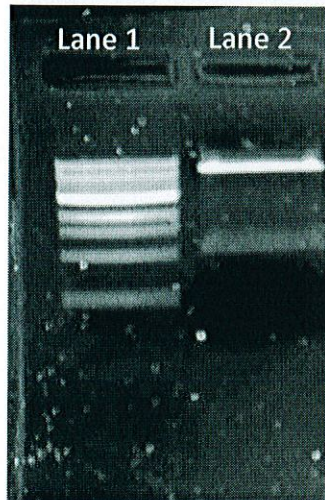


Figure 1

The figure 2 shows the a sample of an agarose gel stained with erthydium bromide with material from

the digestion of the ligation of the KIAA 1362 DNA with that of the pMAL plasmid. The results in Lane 1 are that of the 1 kb ladder and Lane 2 contains the KIAA 1362 protein. The ligation results show a distinct band of digested material consistent with the expected length of the protein.

Figure 2



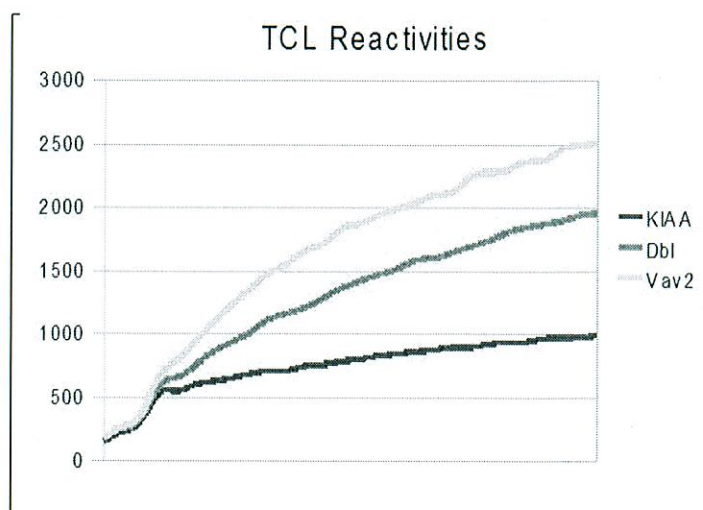
The protein SDS-PAGE gel, displayed in figure 3, dyed with Coomassie blue shows significant bands in the proper location compared to the 1 kb ladder. This confirms that there is a protein that exists of similar size to the potential KIAA 1362 GEF.

Figure 3



The figure 4 below, demonstrates the fluorescent activity of the KIAA 1362 with GTPase TCL. There was some reactivity shown over all but nothing significant like that of Dbp and Vav2. This indicates that

it is likely that there is some factors that are similar, but full activity was not accomplished.



Discussion

The purified GEF KIAA 1362 was found to have no significant catalytic effect to the levels of bound GTP to the tested GTPases TCL, Rac1 and Rho A. There was some noticeable activity with Rho-
Figure 4
family GTPase TCL. Considering its close relation to Cdc42 and TC10, further work may include these proteins in the testing again. TCL is a fairly new protein to the Rho-family and is found primarily expressed in the heart tissue of humans (16). The exact area of expression of KIAA 1362 is still not widely known and the specialization of its only reactive GTPase points to a high chance of specialization for KIAA 1362.

Several factors could have contributed to the low reactivity. The KIAA 1362 protein used here was just a small segment of the entire GEF consisting of only the DH and PH domains and 10 base pairs (bp) on either side. The fact that the FYVE domain was omitted may have significantly contributed to the protein inactivity. The lack of specific bindings of the Zn^{+2} ions and other secondary factors may have resulted in the inactivity. Without the full length of the protein, the activity may be drastically affected. The host the protein was generated within may also have had a drastic effect. The

growth of the protein was recombinantly produced within E. coli BL21 cells, and the protein may need a specific human cellular environment to be properly assembled. Without the specific environment key elements of proper quaternary folding may be lacking. Improper folding can lead to serious malfunctions of the binding site and any necessary conformational changes for activation of the GTPase.

Activity results may have been due to failures with the GTPases tested themselves. GTPases are very sensitive proteins and lab stocks may not have been fresh enough to result in optimum reactivity. Without the optimum freshness the samples could have started to degrade and possibly slip into misformation. Changing assay type by which the exchange of GDP/GTP was measured to a filter type of assay could increase the sensitivity.

Sources

1. Aspenström P, Fransson A & Saras J. *Rho GTPases have diverse effects on the organization of the actin filament system. Biochem J* 2004, 377: 327-337.
2. Takai Y, Sasaki T & Matozaki T. *Small GTP-binding proteins. Physiol Rev* 2001, 81:153-208.
3. Raftopoulou M & Hall A. *Cell migration: Rho GTPases lead the way. Dev Biol* 2004, 265(1):23-32.
4. Whitehead, I., Zohn, I., & Der, J. *Rho GTPase-dependent transformation by G protein-coupled receptors. Oncogene.* 2001, 20(13): 1547-1555.
5. Wherlock, M., & Mellor, H. *The Rho GTPase family: a Racs to Wrchs story. Journal of Cell Science.* 2002, 115: 239-240.
6. Voet D, Voet J G, Pratt C W. *Fundamentals of Biochemistry: Life at the Molecular Level.* New York (NY): Wiley; 2008. 1240 p.
7. Schultz et al. SMART: simple modular architecture research tool. *Proc. Natl. Acad. Sci. USA* (1998). 95, 5857-5864
8. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walters P. *Molecular Biology of the Cell.* New York (NY): Garland Science; 2002. 1616
9. Chhatrivala, M.K., Betts, L., Worthylake, D.K., & Sondek, J. *The DH and PH domains of Trio coordinately engage Rho GTPases for their efficient activation. J Mol Biol.* 2007, 368(5): 1307-1320.
10. Rossman, K.L., et al. Functional analysis of cdc42 residues for guanine nucleotide exchange. *J. Biol. Chem.* 277, 50893-50898 (2002).
11. Orico, A., et al. Phenotypic and molecular characterization of the Aarakog-Scott syndrome, a survey of clinical viability in light of Fgd1 mutation analysis in 46 patients. *Eur. J. Hum. Genet.*

12, 16-23 (2004).

12. Yip Y.L., Scheib H., Diemand A.V., Gattiker A., Famiglietti L.M., Gasteiger E., Bairoch A.

The Swiss-Prot variant page and the ModSNP database: a resource for sequence and structure information on human protein variants Hum. Mutat. 23:464-470(2004).

13. Rossman, K.L., Der, C.J., & Sondek, J. *GEF means go: turning on RHO GTPases with nucleotide exchange factors. Nat Rev Mol Cell Biol.* 2005, 6(2): 167-180.

14. Pasteris, N.G., et al. Isolation and characterization of the faciogenital dysplasia gene, a putative Rho/Rac guanine nucleotide exchange factor. *Cell.* 79, 669-978 (2004).

15. Vignal, E., et al. Characterization of TCL, a new GTPase of the Rho-family family related to TC10 and Cdc42. *Jour Bio Chem* (2000). 275, 36457-36464.

16. Fackler, O.T., Luo, W., Geyer, M., Alberts, A.S., & Peterlin, A.B. Activation of Vav by Nef induces cytoskeletal rearrangements and downstream effector function. *Mol. Cell.* 3, 729-739 (1999).