McNair Scholars Journal

Volume 10 | Issue 1 Article 9

1-1-2006

Exploring Important Protein Interactions in the Mammalian Diaphanous-related Formins: A Closer Look at the Relationship between the Intracellular Skeletal Network and a Single Amino Acid Residue

Brent Hehl
Grand Valley State University

Exploring Important Protein Interactions in the Mammalian Diaphanous-related Formins: A Closer Look at the Relationship between the Intracellular Skeletal Network and a Single Amino Acid Residue

Brent Hehl
McNair Scholar

Dr. Bradley Wallar, Ph.D.
Faculty Mentor

ABSTRACT

The Diaphanous-related formin protein family plays a key role in intracellular cytoskeletal regulation. Due to their far-reaching importance, these proteins must be highly regulated themselves. With recent exploration of regulatory processes of mDia2, a formin protein found in mammals, the established mechanism of control appears to be more complex than previously thought. This project examines the possibility of phosphorylation at critical sites near the protein regions that regulate formin function. Here, we outline our initial screen for discovering these specific residues within mDia2 and their potential for phosphorylation using site-directed mutagenesis.

Diaphanous-related formins (DRFs) interact with the cellular cytoskeleton through their ability to serve as "molecular switching" signals contributing to the formation and stabilization of actin fibers and microtubules. This conserved protein family thus plays a large role in the regulation and control of cytoskeletal process such as cellular division, maintenance of cell shape, and cell movement (1). Due to their far-reaching importance, these proteins must be highly regulated themselves else such human disorders such as deafness and malignant cancers can occur.

The diaphanous inhibitory domain (DID) and the diaphanous autoregulatory domain (DAD), two regions on opposite ends of the protein, play a strong role in mediating the autoregulation of DRFs. In the inactive state, DID and DAD bind through intramolecular interactions to fold in half or "close" the protein (left side, Fig 1). An activated form of Rho-GTPase must bind to the protein's GTPase binding domain (GBD) to "open" the protein (Fig. 1, right side) by disrupting the intramolecular DID-DAD interaction. This exposes the inner domains to the cytosol, allowing these domains to send signals and activate other cellular pathways.

In several mammalian Diaphanous-related formins such as mDia1, mDia2, and mDia3, DID and DAD regions are highly conserved. During the summers of 2004 and 2005, our lab discovered specific residues in DAD important in DID-DAD binding (2). While at that time, specific DID residues functioning in this interaction were unknown, three independent research laboratories recently determined the DID structure (3-5). These groups also found when certain amino acid residues in the DID region (A256 and I259) are replaced with negatively charged residues, the DID-DAD binding interaction significantly decreases. More importantly, a serine residue lays adjacent to these residues

in mDia1, mDia2 and mDia3 (Fig. 2). When phosphorylated, serine residues becomes negatively charged. The conserved presence of serine so close to these residues suggests the mechanism of mDia regulation may have something to do with this serine residue becoming phosphorylated (negatively charged), which would disrupt the intramolecular forces between DID and DAD, thereby 'opening" the protein. Phosphorylation is a very common method in which many cellular proteins are regulated. A bioinformatics computer program independently identified this specific serine residue to be a possible phosphorylation site. In addition, the serine's physical orientation in DID positions the phosphorylation face toward the location where DAD binds to DID.

While preliminary results from our laboratory display promising results at this residue (S272), the potential of similar, undiscovered sites of activity within the mDia protein family that are involved in both regulation and sub-cellular localization capacities may exist. In this study, we compared the genetic sequence of the three mDIA isoforms to locate conserved amino acid residues with a high probability of interacting with other proteins. Ironically, every residue discovered in this search displays potential for phosphorylation by another protein. The initial screen results indicate phosphorylation and subsequent activation of mDia2 may occur at a few select regions, specifically S56, S150/S154, S177/S181, S329/T330, and T1061. For the purposes of this study, T1061 was investigated for any role in the autoregulation of mDia2 (DID-DAD binding).

Materials and Methods

Site-Directed Mutagenesis – Primers were designed by our laboratory and manufactured by Integrated DNA Technologies. The site-directed mutagenesis technique mostly followed the protocol by Stratagene (QuikChange XL II kit). Using polymerase chain reaction (PCR), the mutation-containing plasmids were amplified and transformed into ultracompetent XL-10 Gold *E. coli* cells (Stratagene). After colonization on an LB-ampicillin (100 μg/ml) plate, the resulting colonies were grown in 5 mL cultures at 37°C for 18 h and the plasmid subsequently extracted using the Promega Plasmid Miniprep kit. After the extracted DNA was sequenced at the Van Andel Institute DNA sequencing facility to ensure correct mutagenesis, the bacteria were re-grown in 200 mL cultures and the DNA extracted using the Promega Plasmid Midiprep kit. The midiprepped plasmid DNA was used for microinjection.

Microinjection and Fluorescent Image Acquisition – Mouse fibroblasts (NIH3T3) cells were maintained in Dulbecco's Modified Essential Medium (DMEM; Gibco) containing 10% (v/v) fetal bovine serum (FBS; Gibco) and plated on glass coverslips. Twenty-four h before microinjection, the cells were transferred to DMEM containing 0.1% (v/v) FBS. Coverslips were independently microinjected with wildtype mDia2, T1061G, and T1061E plasmids at a concentration of 100 ng/μL. The cells expressed the injected plasmid for 4 h, followed by fixation with 3.7% formaldehyde for 5 min. A subsequent 1X PBS washing and permeabilization of the cells with Triton X-100 for 5 min prior to 45 min incubation with 1:300 Alexa 488-phalloidin/PBS allows visualization of F-actin. After incubation and 1X PBS washing, the coverslips were mounted on microscope slides using gelvatol as a sealant. All images were acquired on a Olympus BX51 upright research microscope using either a 66X or 100X oil objective.

Results

Mutant T1061 mDia2 has been successfully expressed in mouse fibroblasts – Successful microinjection of specific mDia2 mutants into mouse fibroblasts have been shown to sufficiently disrupt DID-DAD binding to the point of significantly changing the cell's phenotype as compared to fibroblasts injected with normal, "inactive" mDia2. We were also able to microinject fibroblasts with the two mutants of T1061 (T1061G and T1061E) as well as the normal-type mDia2 (Fig. 3). While the data from this initial screen show limited phenotypic changes, more experimentation needs to be done to more fully understand and determine T1061's role in mDia regulation.

In this experiment, we would expect to see activation of mDia2 with the T1061E mutation, similar to S272D (Fig. 3). If phosphorylation occurs, a cell microinjected with T1061E would be expected undergo vast changes in physiology. Unfortunately, the technique we used here has limitations; there are still biochemical assays to be performed to more fully understand T1061s role in mDia2.

Discussion

The DNA sequence of mDia2 was scanned in the online database MotifScan to determine the probability of potential protein interaction sites. The resulting scores and their locations in mDia2 were then compared against the aligned sequences of mDia1 and mDia3 in order to determine the overall level of residue conservation. To further refine the search, the sites were then located in the known protein crystal structure for judging if the site had the potential to interact with another protein.

Several factors contributed to the selection of T1061 for further analysis. Of the five chosen protein sites, only this one appears in the DAD region. This is significant when put into context with experiments performed by Higgs (7). His work suggests that GTPase binding may not be the only signal

present in mDia regulation. Based of his results and evidence of phosphorylation within mDia, we propose that GTPase binding may be the catalyst for the *initial* disruption of DID-DAD binding while a second signal, through means of a phosphorylation event somewhere near the DID and DAD regions, is necessary to prolong the duration of mDia activation. T1061, in the DAD region, appears a likely site for this event to occur with the GTPase "activating" signal being bound in the DID region.

The potential kinases likely to cause this phosphorylation event at T1061 contribute to this site's selection as well. Some of these kinases have been found at the tips of actively elongating actin filaments similar to where activated mDia2 localizes. With successful microinjection of GFP-labeled mDia2 mutants designed specifically to test the possibility of phosphorylation, we should see a signal at the growing ends of actin filaments.

The amino acid mutants chosen imitate both non-phosphorylated (glycine; G) and phosphorylated (glutamic acid; E) variants of threonine. Mutants like these eliminate the ability of the cell to utilize phosphorylation as signaling switch. Now the protein has been "hardwired" into either a mimicked phosphorylated (T1061E) or non-phosphorylated (T1061G) state, thereby bypassing any potential signaling pathways present in the cell.

Figure 1. *Regulation of the Diaphanous-related Formins*
Two regions, DID and DAD, account for the formins' ability to autoregulate themselves. These two domains on opposite ends of the protein bind together in the "inactive" form. This is the natural protein conformation. When an active Rho-GTPase binds to DID, the DID-DAD binding interaction disassociates, thereby opening the protein and exposing the inner domains to the cytosol where they mediate cytoskeletal processes.

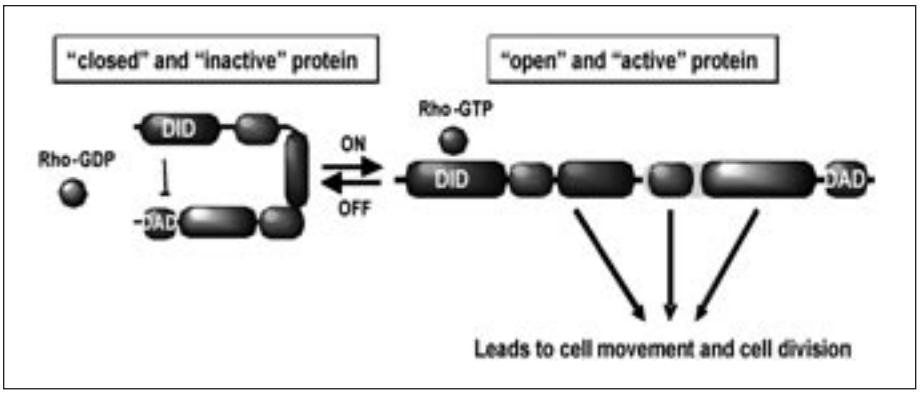


Figure 2. *Conserved Serine Residue in DID*
Two conserved residues demonstrated as very important to DID-DAD binding (A256 and I259) also have a neighboring serene residue found across all three isoforms of mDia as well. When A256 and I259 are replaced with negatively charged residues, the binding interaction drastically decreases. This serene appears to be a convenient place for a phosphorylation event to occur, an event where the cell can selectively add and remove a large, negatively charged phosphate group.

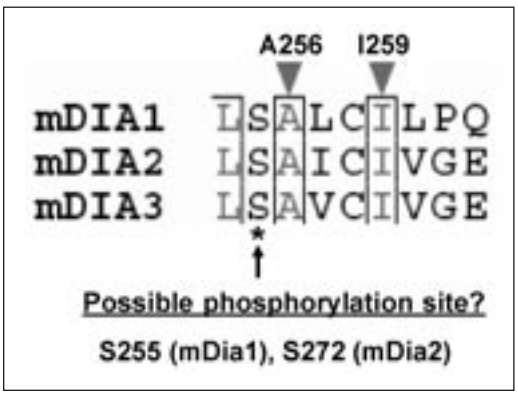
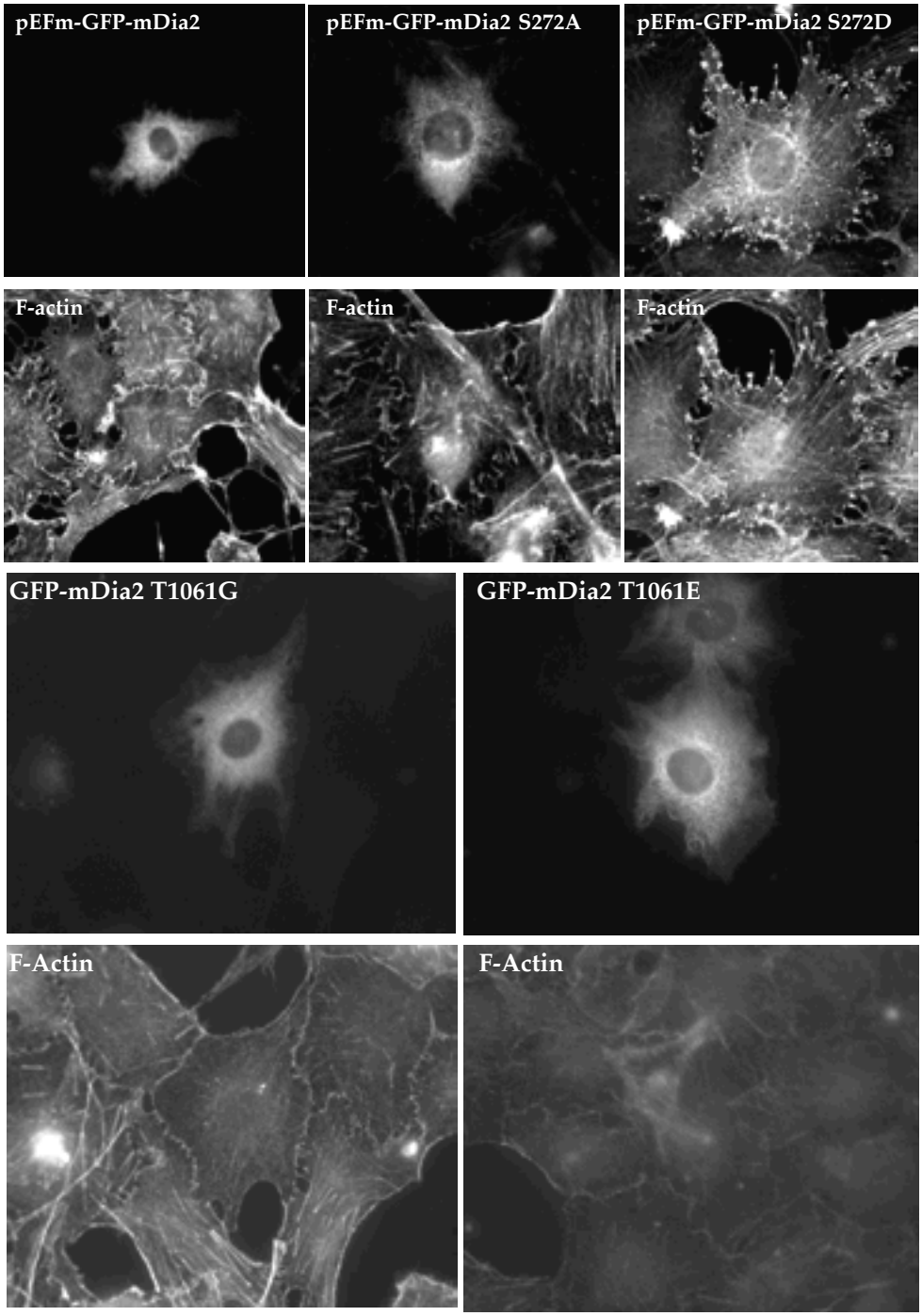


Figure 3. *Results*
Wild-type mDia2 labeled with GFP localizes in the cytoplasm with no marked changes in cell morphology; it's inactive. We expect and see the same for S272A (lab results obtained 2005) and in T1061G, as these mutations mimic a constantly non-phosphorylated state. In S272D, the cell morphology undergoes wide changes. Filipodia and other such protrusions emanate from the cell. mDia2 localizes at the tips of these protrusions and large amounts of actin can be found there as well. In T1061E, we don't see much change from T1061G, but this technique is only one tool available, with all its advantages and limitations, to determine if phosphorylation can occur. More experiments need to be run to more fully understand the relationship between T1061 and phosphorylation.



References

1. Wallar, B. J. and Alberts, A. S. (2003) *Trends Cell Biol*. **13**(8):435-446.
2. Wallar, B. J., Stropich, B. N., Schoenherr, J. A., Holman, H. A., Kitchen, S. M., and Alberts, A. S. (2006) *J. Biol. Chem*. **281**:4300-4307.
3. Lammers M., Rose R., Scrima A., and Wittinghofer A. (2005) EMBO J. **24**:4176-87.
4. Otomo T., Otomo C., Tomchick D.R., Machius M., and Rosen M.K. (2005) *Mol. Cell* **18**:273-81.
5. Nezami AG, Poy F, and Eck MJ. (2006) *Structure* **14**:257-63.
6. Ghanekar, Y. and Lowe, M. (2005) *Trends Cell Biol*. 15:511-514.
7. Li, F. and Higgs, H.N. (2005) *J. Biol. Chem*. **280**(8):6986-92.