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13. ABSTRACT (Maximum 200 Words)

Improper regulation of the level and duration of activated erbB family growth factor receptors at the cell surface can lead to uncontrolled cell proliferation and transformation. The GTPase dynamin is a regulator both of transport of receptors to the plasma membrane and down-regulation of receptors via receptor-mediated endocytosis (RME). The localization of specific dynamin isoforms led us to hypothesize that different dynamin isotypes are involved in the distinct processes of vesicle budding from the TGN and receptor-mediated endocytosis from the plasma membrane. This proposal outlines a study of the role of different dynamin isoforms in cellular trafficking to define which members of the dynamin family are involved in up- versus down-regulation of erbB receptors. The analysis of these hypotheses is still on going. I also hypothesized that the pleckstrin homology (PH) domain of dynamin is essential for dynamin targeting. My studies indicate that this is not the case, and that the PH domain may instead be directly involved in vesicle scission. PH domains have characteristics that suggest they will be good targets for small molecule drugs, so understanding the role of the PH domain in dynamin function should allow for modulating dynamin activity to control the level of erbB-mediated signaling.

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Introduction

Improper regulation of the level and duration of activated erbB family growth factor receptors at the cell surface can lead to uncontrolled cell proliferation and transformation via over-stimulation of mitogenic signaling cascades. The large GTPase dynamin is a key regulator both of transport of receptors to the plasma membrane after receptor biosynthesis and down-regulation of receptors via receptor-mediated endocytosis (RME). Disruption of RME has been shown to render the epidermal growth factor receptor (erbB1) oncogenic (1), illustrating the importance of proper attenuation of signaling by down-regulation. Dynamin exists as a family of proteins, with four splice variants of the ubiquitious dynamin-2 isoform being expressed in all cell types. Previous studies have suggested that different splice variants possess unique patterns of subcellular localization, which may be linked to their participation in distinct trafficking pathways (2). This proposal outlined experiments to systematically determine the localization of each variant, and their differential roles in the key processes of receptor up- (via delivery of new receptors to the cell surface) and down-regulation (via RME). This proposal also addressed the mechanistic role of the pleckstrin homology (PH) domain in dynamin function, which may provide a pharmacologic target for modulating dynamin activity.

Body

This is the current status of progress compared with the Statement of Work.

Task 1: Determine the subcellular localization of the four dynamin-2 splice variants.

a. Clone GFP-fusions of each dynamin-2 splice variant (months 1-5)
b. Isolate Clone 9 cell lines stably expressing each GFP-fusion (months 5-10)
c. Determine subcellular localization of each variant (months 11-15)

Task 1a: The cloning of the four dynamin splice variants proved to be more difficult than anticipated. Given the information from previous publications indicating the ubiquitous expression of all variants, we attempted to clone out each variant by RT-PCR from HeLa cell RNA. We were successful in pulling out the dynamin-2 (ba) splice variant easily, but found the other variants to be represented at much lower levels. Despite extensive screening including preparing RNA from several sources, we were unable to obtain the other variants. We plan to circumvent this problem (and expedite Task 1b) by obtaining both the constructs and stable clone 9 cell lines for the (aa) and (ab) variants from the laboratory of Mark McNiven at the Mayo Clinic. The remaining variant (bb) can then be easily constructed from either the (ab) clone or by engineering a simple deletion. Dynamin-2 (ba) was successfully cloned as N- and C-terminal GFP fusions, and expression of the GFP-fusion was verified by light microscopy.

Task 1b: Dynamin-2 (aa) and (ab) stable cell lines will be obtained from the laboratory of Mark McNiven at the Mayo Clinic. Once the (bb) variant is in hand, stable cell lines of GFP-dynamin (ba) and (bb) will be isolated.

Task 1c: Not yet initiated.

Task 2: Demonstrate that the distinct subcellular localization demonstrated in Task 1 is mirrored by the role of each splice variant in cellular trafficking.

a. Clone each dynamin-2 splice variant of interest into tet-regulatable vector pUHD (months 15-18)

b. Determine the effect of dominant-negative mutants of relevant splice variants on receptor-mediated endocytosis (months 18-22)

c. Determine the effect of dominant-negative mutants of relevant splice variants on biosynthetic transport to the plasma membrane (months 23-28)

d. Perform *in vitro* TGN-budding assays with dynamin-2 variants (months 29-36)

Task 2: This task does not begin until into the second year. The clone of GFP-dynamin 2 (ba) has been cloned into pUHD (task 2a), and the remainder has not yet been initiated.

Task 3: Test the hypothesis that multivalent PH domain-mediated interactions are required for targeting of dynamin to clathrin-coated pits.

a. Purify recombinant wild-type and mutant dynamins (months 1-5)

b. Perform GTPase assays to test the effect of PH mutant dynamin on Wt dynamin (months 6-12)

c. Perform localization and internalization assays on the PH/T65A double mutant (months 18-30)

Task 3a: This task has been successfully completed. Both wild-type and the PH domain mutant (PH*) of dynamin were highly purified from baculovirus-infected Sf9 cells at a yield of 2 milligrams per liter. A SDS-PAGE gel of these purified proteins is shown in Figure 1. There is a small amount of degradation that results in clipping of the extreme C-terminus of dynamin (in the proline-rich region), but does not affect activity in our assays.

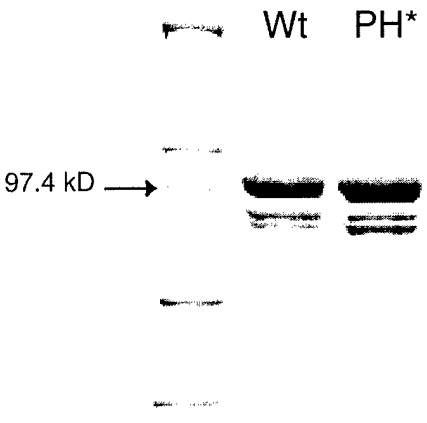

Figure 1. Purified dynamin Wt and PH* mutant (each approximately 100 kD) as analyzed by SDS-PAGE

Task 3b: This task has also been completed, and also furthered by some additional methods to probe the effect of PH* mutant dynamin on Wt dynamin.

First, the ability of vesicles containing the phosophoinositide PIP_2 to stimulate the GTPase activity of Wt and PH* dynamin was analyzed. As expected, the PH* mutant was essentially unable to undergo stimulation of GTPase by these vesicles (Figure 2D), despite the finding that induction of phosphoinositide-independent activation by low salt activated PH* as well as Wt (Fig. 2B).

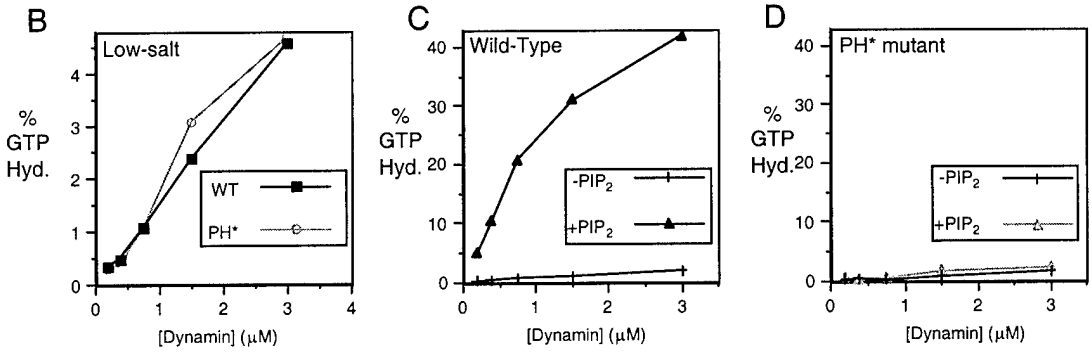

Figure 2. GTPase assays of Wt and PH* mutant dynamin. In panel B the GTPase activity of dynamin was stimulated by assembly due to dilution in low salt. The similarity of Wt and PH* here indicates that the PH* mutation does affect intrinsic GTPase. Panel C illustrates the degree of GTPase stimulation of Wt dynamin with PIP_2-containing vesicles. The PH* mutant fails to be stimulated (Panel D).

Next, the influence of molar excesses of the PH* mutant on Wt GTPase was investigated. Excesses of the PH* mutant were incubated with Wt dynamin prior to performing the GTPase assay. Although PH* appeared to diminish the PIP_2-stimulated GTPase activity of Wt dynamin, this effect plateaued, leaving approximately 50% of the activity of Wt dynamin alone (Figure 3).

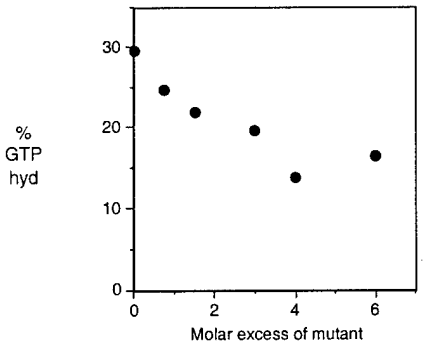

Figure 3. Addition of molar excesses of PH* mutant dynamin to a constant concentration of Wt dynamin causes an approximately 50% decrease in PIP_2-stimulated GTPase.

Given this marginal effect, we looked for an alternative assay with which to address this question. We therefore employed surface plasmon resonance (SPR) as a read-out of the recruitment of dynamin to a PIP_2 surface. Again, we first characterized the ability of dynamin to interact with this surface directly. Again, the PH* dynamin proved to be deficient in binding to PIP_2 (Figure 4).

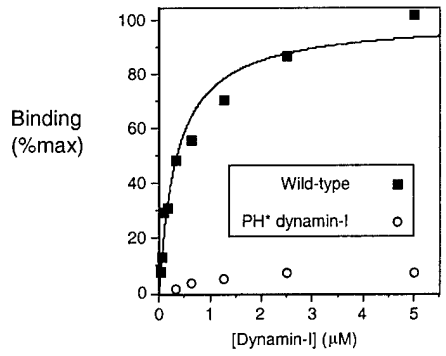

Figure 4. Wild-type dynamin, but not PH* mutant dynamin, binds to a PIP_2 surface by SPR using BiaCore.

We mixed PH* dynamin with wild-type dynamin in order to look for a dominant-negative effect on binding by the wild-type protein. The result closely mirrored that seen in the GTPase assay (Figure 3), where PH* mutant dynamin caused only a maximum of 50% loss of binding by Wt dynamin (Figure 5).

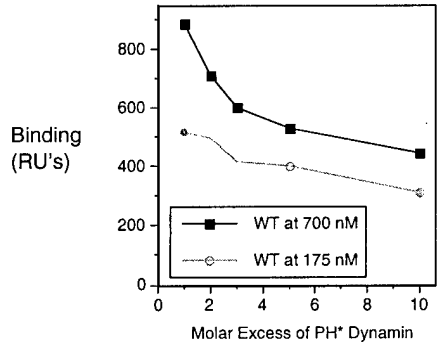

Figure 5. Addition of excess PH* mutant to Wt dynamin has a minimal effect at low concentrations (gray), and at most an inhibitory effect of approximately 50% at higher concentrations (black).

The most logical conclusion from these studies is the following: The maximum 50% dimunition of both wild-type binding to PIP_2 and PIP_2-stimulated GTPase by excess PH* mutant is unlikely to explain the potent *in vivo* dominant-negative effect of this mutant. This indicates that the dominant-negative effect of PH* dynamin on RME is likely not due to mistargeting of endogenous dynamin. Therefore, it is likely that the PH domain of dynamin is not involved in targeting of the molecule, but rather in some other aspect of endocytosis. This will be directly investigated in the future in Task 3c.

Key Research Accomplishments

- Cloning of the dynamin-2 (ba) variant as a GFP-fusion, and subcloning into the tet-regulatable pUHD vector
- Purification of Wt and PH* mutant dynamin from baculovirus-infected Sf9 cells
- Characterization of the Wt and PH* dynamins in low-salt and PIP_2-stimulated GTPase assays
- Demonstration that the PH* mutant of dynamin can only diminish approximately 50% of PIP_2-stimulated GTPase by Wt dynamin
- Characterization of the binding properties of Wt and PH* dynamin to a PIP_2 surface via SPR

- Demonstration that the PH* mutant of dynamin can only diminish approximately 50% of PIP_2 binding by Wt dynamin

Reportable Outcomes

Poster: Lee, A., **King, M.C.**, Lemmon, M.A. "The role of the dynamin PH domain in endocytosis". Gordon Research Conference: Protein Phosphorylation and Second Messsengers, June, 2002.

Publication: **King, M.C.**, Raposo, G, Lemmon, M.A. "The Dynamin-like GTPase MxB Participates in Regulation of Nucleocytoplasmic Trafficking", *in submission.*

Conclusions

Our initial studies indicate that the PH domain of dynamin is not involved in targeting this protein to its location of action at the plasma membrane, specifically at forming clathrin-coated pits. As dynamin with a mutated PH domain acts as a dominant-negative inhibitor of receptor-mediated endocytosis in cells, this indicates that the PH domain is critical to dynamin function, but with a novel mechanism. Our further studies intend to investigate this further, with the hopes that understanding the role of the PH domain may lead to the identification of this module as a pharmacologic target. Although progress has been hampered in obtaining the biological reagents we need in order to investigate the differential roles of dynamin-2 splice variants in receptor up- and down- regulation, we expect to move on to the characterization phase of this task soon as we will obtain some of these reagents from colleagues. The combination of the knowledge obtained thus far and the characterization to come will provide us with the background needed to design a system wherein modulation of specific dynamin isotypes will allow us to compensate for altered erbB receptor expression profiles that are implicated in cancer.

References

1. Vieira, A., Lamaze, C. & Schmid, S. (1996) *Science* **274,** 2086-2089.
2. Cao, H., Garcia, F. & McNiven, M. (1998) *Mol. Biol. Cell* **9,** 2595-2609.