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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 recptor biosynthesis and down-regulation of receptors via receptor-mediated endocytosis (RME), during which it is involved in the scisson of endocytic vesicles. 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. This proposal addresses the mechanistic role of the pleckstrin homology (PH) domain in dynamin function, which may provide a pharmacologic target for modulating dynamin activity. The PH domain binds phosphatidylinositol (4,5) bisphosphate (PI(4,5)P2) at the plasma membrane (PM), but the role of this binding is not yet understood. The experiments detailed below address whether PI(4,5)P2 binding is involved in targeting of dynamin to the PM, or whether phosphoinositide binding instead plays a more physical role in the scission of endocytic vesicles, and therefore receptor downregulation.

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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), during which it is involved in the scission of endocytic vesicles. 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. This proposal addresses the mechanistic role of the pleckstrin homology (PH) domain in dynamin function, which may provide a pharmacologic target for modulating dynamin activity. The PH domain binds phosphatidylinositol (4,5) bisphosphate ($PI(4,5)P_2$) at the plasma membrane (PM), but the role of this binding is not yet understood. The experiments detailed below address whether $PI(4,5)P_2$ binding is involved in targeting of dynamin to the PM, or whether phosphoinositide binding instead plays a more physical role in the scission of endocytic vesicles, and therefore receptor downregulation.

Body (please refer to attached figures)

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: This task is now complete. A clone of the GFP-fusion of the splice variant (ba) was generated in the first year. We now have clones of all four dynamin splice variants as GFP-fusions, including (aa), (ab), and (bb).

Task 1b: This task is partially complete, as we now have stable cell lines for GFP fusions of the variants (ba), (aa), and (ab). The (bb) variant remains to be completed.

Task 1c: Consistent with the published literature, variant (ba) was found to be at the plasma membrane (2), variant (aa) was present at the TGN and the plasma membrane (3) and variant (ab) was found at only the plasma membrane (3). The localization of variant (bb) will be initiated after a suitable stable cell line is isolated.

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 2a: This aim has been initiated, but not yet completed as the Tasks 2b, 2c, and 2d require the isolation of stable expressors in a cell line with the tet-activator/repressor (tTA cell lines, in this case tTA HeLa cells) after completion of the necessary cloning, which was not initially outlined in the proposal. In addition, each cell line must maintain its tet-regulation, making isolation of these lines more difficult. To date cell lines expressing Wt and mutant dynamin-2 (ba) have been isolated, and generation of the additional cell lines is in progress. This should be complete within the next six months.

Task 2b: The effect of the K44A and PH* mutations for the (ba) variant on receptor-mediated endocytosis have been initiated. Expression of both the K44A and PH* dynamin mutants were found to disrupt RME, as expected since this splice variant localizes to the plasma membrane.

Task 2c and 2d: Not yet 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 was completed in the first year, and additional dynamin mutant proteins utilized as controls below were cloned, expressed, and purified similarly.

Task 3b: Although initial results for this task were obtained in the first year, additional experiments have revealed that the ability of PH mutant dynamin (PH*) to act as a dominant-negative inhibitor of Wt dynamin *in vitro* is more potent than originally believed. The marginal effect documented in the previous report was found to be an artifact of protein storage conditions, and the results obtained with freshly purified protein are described below.

As initiated previously, we began using surface plasmon resonance (SPR) to address directly the effect of the PH* mutant on Wt dynamin binding to its ligand $PI(4,5)P_2$. This is a more direct assay than the PIP_2-stimulated GTPase assay originally proposed. In addition we have included two control proteins for the effects of mutant dynamin on Wt dynamin binding. This includes K44A dynamin, which is deficient in nucleotide binding but should bind phosphoinositides similarly to Wt dynamin. An additional mutant, PH*/ΔCC, has the PH* mutation of the original PH mutant dynamin and a deletion of a critical coiled-coil sequence in the assembly domain that prevents multimerization. The addition of this coiled-coil deletion should revert the dominant-negative activity of the PH* mutant on Wt dynamin, as it cannot multimerize with the Wt protein.

The binding constants for each of these proteins to PIP_2 as evaluated by SPR were as predicted (Fig. 1A). Wt and K44A dynamin have similar binding affinities (around 200 nM), whereas both the PH* and PH*/ΔCC dynamin mutants are extremely deficient in PIP_2 binding with Kds in excess of 20 μM.

We next investigated the binding of mixtures of Wt and mutant dynamin proteins at a constant total protein concentration (2 μM). The results of these experiments are presented in Figure 1B. As Wt and K44A dynamin are equivalent in their binding to PIP_2, we expect that the binding should remain constant regardless of the ratio of Wt and mutant proteins. This was indeed found to be the case. For the PH*/ΔCC mutant, which cannot bind to PIP_2 efficiently, we expect the binding to slowly diminish as a larger proportion of the 2 μM protein is represented by the mutant. However, this is simply a dilution of Wt binding affinity as compared to the effect of PH* dynamin, which cannot bind PIP_2, but also inhibits binding of Wt dynamin. It is obvious from the effect of PH* versus the PH*/ΔCC that the ability of PH* to oligomerize with Wt dynamin is required to decrease the avidity of Wt dynamin for PIP2, as represented by the highly potent effect of PH* dynamin.
This result was further investigated by mixing increasing ratios of mutant dynamin at a constant Wt dynamin concentration (400 nM). As seen in Figure 1C, these results are consistent with the conclusions above. Addition of K44A dynamin increases the total binding, as this mutant binds PIP_2 efficiently. There is only a slight effect of adding excess PH*/ΔCC dynamin, consistent with the inability of this mutant to oligomerize with Wt dynamin and affect its binding. Only PH* dynamin significantly disrupts Wt binding in a dose-dependent fashion. The efficacy of the PH* mutant dynamin is approximately analogous to the potency of this mutant at inhibiting RME *in vivo*, indicating that it is highly plausible that PH* dynamin exerts its effect by disrupting the avidity of PIP_2 binding by endogenous dynamin in the cell. This validates the hypothesis of Task 3. These results will be confirmed in the next year using the GTPase assay originally proposed.

Task 3c: This task is largely complete. The PH* mutation appears to affect neither the targeting nor inhibition of RME by the GTPase-deficient T65A mutant. In Figure 2A, T65A dynamin is found to colocalize at trapped coated pits/tubules with GFP-clathrin. Deletion of the proline/arginine-rich domain responsible for initial dynamin targeting via the SH3 domain-containing protein amphiphysin disrupts this targeting (seen as a lack of colocalization of T65A/ΔPRD dynamin with clathrin). However, introduction of the PH* deletion (T65A/PH*) does not disrupt colocalization, indicating that phosphoinositide binding by the PH domain is not required for dynamin targeting. Therefore, the effect of PH* dynamin exhibited in Task 2 must occur at a stage of endocytosis after dynamin targeting. As anticipated, T65A/PH* dynamin inhibits RME similarly to T65A dynamin (Figure 2B). We have therefore gone on to address the effect of PH* dynamin at later stages of endocytosis, which currently includes analysis of lipid tubulation and GTP-dependent constriction of lipid tubules as assessed by negative-stain electron microscopy. Preliminary results indicate that although PH* dynamin is capable of tubulating and constricting lipids as Wt dynamin, it does so with much reduced efficiency. Although our study is not yet complete, it appears likely that the PH domain is critical for exerting pressure on the lipids at the neck of invaginated coated pits during constriction.

Key Research Accomplishments

- Cloning of the dynamin-2 splice variants as GFP-fusions.
- Generation of stable cell lines for expressing GFP-fusions for three of the four splice variants.
- Cloning of several Wt and mutant GFP-fusions into the pUHD vector for tet-regulated expression
- Generation of tet-regulatable stable cell lines expressing dynamin mutants
- Validation of the hypothesis stated in Task 3, by demonstrating the dominant-negative effect of PH-mutant dynamin on phosphoinositide binding by Wt dynamin, including key control experiments with other dynamin mutants
- Demonstration that introduction of the PH domain mutation does not prevent targeting of T65A dynamin to the necks forming clathrin-coated pits

Reportable Outcomes:

Publications:

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

King, M.C., Kelley A. Bethoney, Anthony Lee, Graça Raposo, Jenny E. Hinshaw, and MarkA. Lemmon. "Role of the Dynamin Pleckstrin Homology Domain in Receptor-Mediated Endocytosis", *in preparation.*

Conclusions

We have now assembled almost all the reagents necessary to address the role of different dynamin isotypes in trafficking of receptors to and from the plasma membrane. We have acquired data consistent with our hypothesis stated in Task 3 (that PH* dynamin is a dominant-negative inhibitor of phosphoinositide binding by Wt dynamin *in vitro*). We also now know 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. Preliminary data suggests that the PH domain may function during the constriction of the neck of nascent vesicles. 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.

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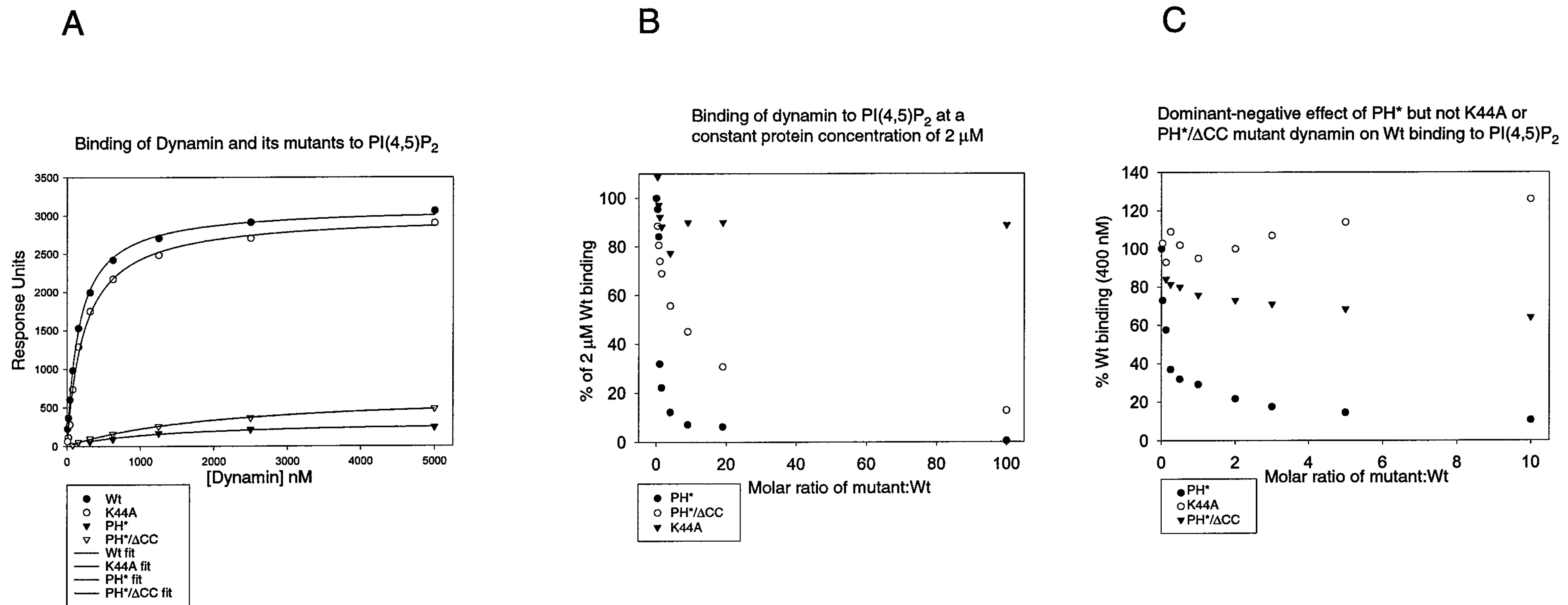

Figure 1. Analysis of Wt and mutant dynamin binding to $PI(4,5)P_2$ by surface plasmon resonance. A. Wt and K44A dynamin bind with similar Kds (around 200 nM), while the PH* mutant and PH*/ΔCC double mutant bind very weakly (> 20 μM). B. Addition of excesses of mutant dynamin to Wt dynamin at a constant protein concentration demonstrates that PH* dynamin exerts a dominant-negative effect on Wt binding and must be capable of oligomerization to exert this effect. C. Only molar excesses of PH* dynamin can disrupt Wt binding efficiently.

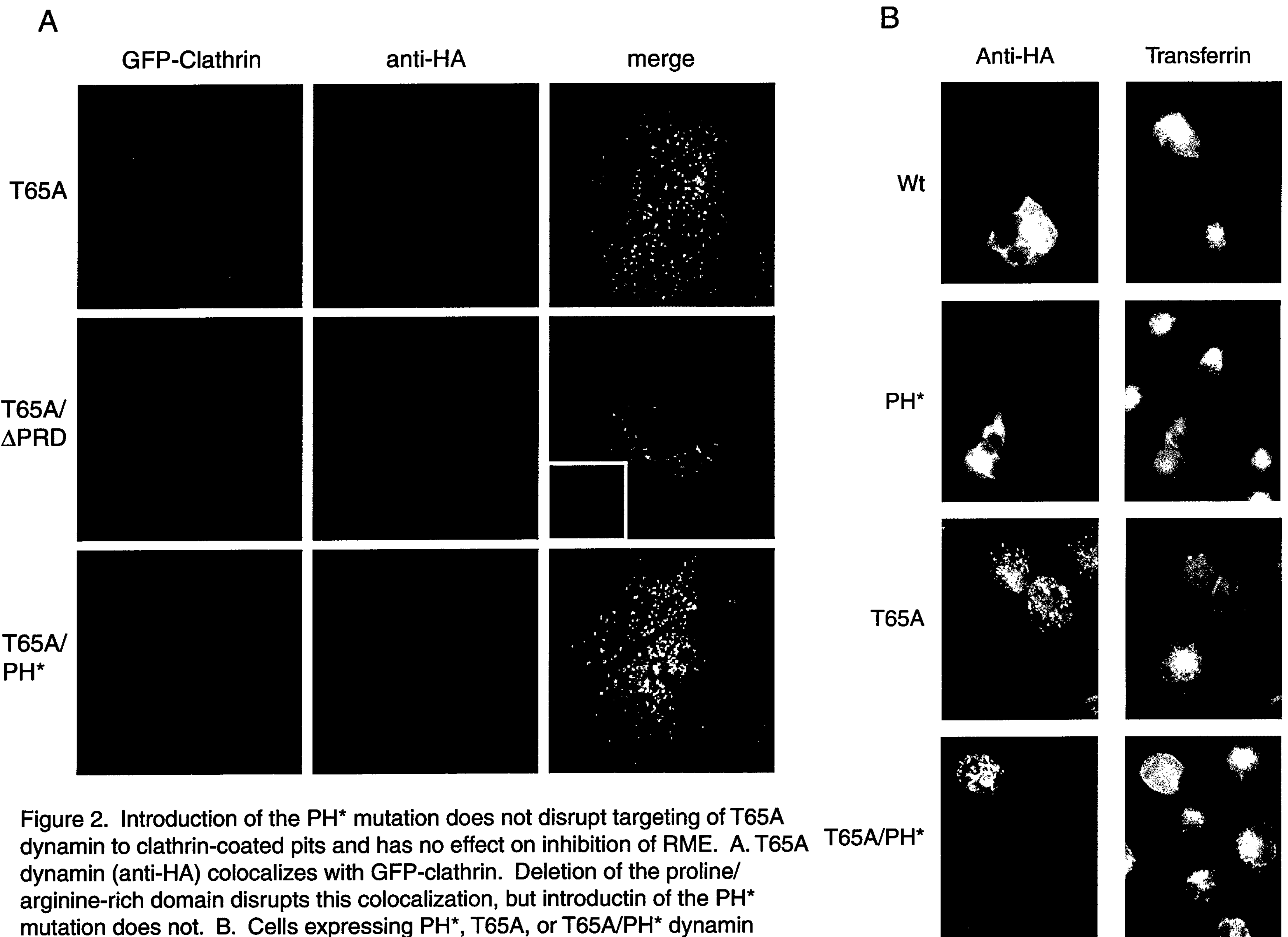

Figure 2. Introduction of the PH* mutation does not disrupt targeting of T65A dynamin to clathrin-coated pits and has no effect on inhibition of RME. A. T65A dynamin (anti-HA) colocalizes with GFP-clathrin. Deletion of the proline/ arginine-rich domain disrupts this colocalization, but introductin of the PH* mutation does not. B. Cells expressing PH*, T65A, or T65A/PH* dynamin (anti-HA) cannot take up labeled transferrin (a marker for RME), but expression of Wt dynamin has no effect. Internalized transferrin appears as punctate intracellular staining.