Differential SHIP2 Activation by EGF and Insulin Uncovers Pathway-Specific Control of PI(3,4)P_2 Signaling

PhD thesis

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1. Introduction

Phosphoinositides (PIPs) are low-abundance membrane lipids that undergo rapid interconversion and define membrane identity while acting as second messengers. At the plasma membrane, class I PI3Ks convert $PI(4,5)P_2$ to PIP_3, which recruits effectors such as Akt to control survival, growth, metabolism and cytoskeletal dynamics. PIP_3 can be reversed to $PI(4,5)P_2$ by PTEN or converted to $PI(3,4)P_2$ by 5-phosphatases, such as the SHIP enzymes. Once viewed as a PIP_3 breakdown product, $PI(3,4)P_2$ is now recognized as an active molecule with roles in polarity, migration and endocytosis. $PI(3,4)P_2$ can also arise via class II PI3Ks from PI4P, a pathway that is important for endocytosis. Dysregulated PIP metabolism is implicated in cancer and metabolic disease.

As $PI(3,4)P_2$ is recognized as a distinct second messenger, defining the enzymes that control its synthesis and turnover is critical. At the plasma membrane, the 5-phosphatases SHIP1 (hematopoietic-restricted) and SHIP2 (broadly expressed) convert PIP_3 to $PI(3,4)P_2$. SHIP dysregulation has been linked to Alzheimer's disease and metabolic disorders. In cancer, their effects are context-dependent: lowering PIP_3 can be tumor-suppressive, yet the product $PI(3,4)P_2$ can activate Akt isoforms and potentially support oncogenesis. Accordingly, SHIP

enzymes are under active investigation as therapeutic targets, including inhibitor strategies.

Pleckstrin homology (PH) domains are ~120 amino acid long segments found in many signaling proteins. By recognizing specific phosphoinositides, they target proteins to defined membrane regions and thereby provide spatial control over signaling. Their specificity for PIPs has made PH domains widely used live-cell biosensors for tracking phosphoinositide dynamics.

For many PH domains, lipid binding alone does not ensure correct membrane targeting. Proper localization often requires additional protein associations. When isolated PH domains are overexpressed, they can sequester lipids or binding partners and inhibit endogenous protein signaling (a dominant-negative effect). Mutations that preserve lipid binding but disrupt these protein interactions abolish this inhibition. Because PH domains also bind membrane proteins, PH-based biosensors do not report lipid dynamics in isolation, but rather their signals are confounded by non-lipid interactions with the probe, an issue we addressed in this study.

2. Objectives

We defined the following objectives, which fall into two groups:

Investigation of the dynamics and regulation of $PI(3,4)P_2$ production in response to EGF and insulin signaling:

1. To compare the plasma membrane dynamics of PIP_3 and $PI(3,4)P_2$ following stimulation with EGF and insulin in mammalian cells.
2. To elucidate the enzymatic mechanisms governing $PI(3,4)P_2$ production at the plasma membrane, with a specific focus on the role and contribution of SHIP2.
3. To explore the mechanism of EGF induced-SHIP2 phosphorylation.

Optimizing PH domain-based biosensors for improved detection of plasma membrane PIP_3:

1. To evaluate the impact of targeted mutations that disrupt non-lipid interactions on the localization and performance of Akt and GRP1 PH-domains.
2. To adapt the novel biosensors for compatibility with diverse experimental methodologies, including live-cell imaging and BRET-based approaches.

3. Methods

3.1. Confocal microscopy

We used live-cell confocal fluorescence microscopy to visualize the subcellular localization of fluorescently tagged PH-domain probes in cultured cells. Cells were transiently transfected, allowed to express the constructs and serum-depleted before imaging in a physiological buffer. Imaging captured membrane translocation and distribution changes before and after stimulation. Images were processed using only linear adjustments.

3.2. Bioluminescence resonance energy transfer (BRET) measurements

We used live-cell BRET to quantify plasma-membrane phosphoinositides in cultured HEK293A. Cells were transiently transfected with a luciferase donor and a fluorescent acceptor, then measured in a plate reader after adding the luciferase substrate to record real-time emission ratios during agonist stimulation. The BRET signal (530/480 nm) was normalized to the pre-stimulus baseline. Cells expressing only the luciferase donor (no acceptor) defined the 0% BRET level. For sensor-expressing cells, the pre-stimulus ratio was set to 100%, and responses were reported as percent change from baseline, which

minimizes effects of variable sensor expression on absolute ratios. Experiments were conducted in biological replicates.

3.3. Immunoblotting

Cells were serum-depleted, stimulated with agonists and lysed. Proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with antibodies against phosphorylated and total forms of the investigated proteins. Signals were detected by chemiluminescence and quantified by densitometry (ImageJ). Total protein served as the loading control. Phosphorylation sites are shown in brackets, and molecular weights (in kDa) are marked with arrows.

3.4. Mathematical and statistical analysis

Statistical analyses were performed in SigmaPlot 10.0. Data were tested for normality and equal variance. When these assumptions held, we used t-tests or ANOVA with appropriate post-hoc tests. When assumptions were not met, non-parametric alternatives (e.g., ANOVA on Ranks) were applied.

4. Results

4.1. Effect of EGF and insulin stimulation on PIP_3 and $PI(3,4)P_2$ levels in HEK293A cells

To compare receptor-specific phosphoinositide patterns at the plasma membrane, we quantified PIP_3 and $PI(3,4)P_2$ dynamics in HEK293A cells by live-cell BRET using three PIP_3 biosensors (Btk-PH, GRP1-PH, Btk-2xPH) and a $PI(3,4)P_2$ biosensor (TAPP1-2xPH). Cells were stimulated with EGF (100 ng/mL) or insulin (70 nM, 0.7 μM). Both ligands elevated PIP_3, but EGF drove a proportionally larger increase in $PI(3,4)P_2$. Raising insulin concentration amplified both lipids without altering this relationship. Accordingly, the $PI(3,4)P_2/PIP_3$ ratio was consistently higher with EGF across the sensors. High-temporal-resolution BRET after EGF revealed a brief initial dip in the ratio followed by a sustained rise.

4.2. Dissecting the role of SHIP2 and class II PI3Ks in the EGF-induced PIP profile

To identify the source of EGF-evoked $PI(3,4)P_2$ at the plasma membrane, we selectively depleted SHIP2 or class II PI3Ks and monitored PIP_3 (GRP1-PH) and $PI(3,4)P_2$ (TAPP1-2xPH) by BRET. SHIP2 knockdown modestly increased PIP_3 but markedly reduced $PI(3,4)P_2$, lowering the $PI(3,4)P_2/PIP_3$

ratio. Class II PI3K knockdown produced the opposite trend, enhancing $PI(3,4)_2$ and raising the ratio. Together, these data indicate that, after EGF stimulation, $PI(3,4)P_2$ at the PM is generated primarily by SHIP2 dephosphorylation of class I PI3K-derived PIP_3, consistent with the observed early dip and subsequent rise in the $PI(3,4)P_2/PIP_3$ ratio.

4.3. SHIP2 phosphorylation dynamics in response to EGF and insulin

To probe how SHIP2 is regulated downstream of the investigated RTKs, we compared its phosphorylation after EGF versus insulin stimulation. Following 5 h serum starvation, cells were stimulated for 5 min with EGF (100 ng/mL) or insulin (70 nM or 0.7 μM) and analyzed by Western blot. EGF induced robust SHIP2 phosphorylation at Tyr986/987 and Tyr1135, whereas insulin failed to elicit detectable phosphorylation even at the higher dose.

4.4. PLCγ-induced calcium signal regulates SHIP2 phosphorylation in EGFR pathway

To define upstream drivers of EGF-evoked SHIP2 phosphorylation, we first chelated intracellular calcium with BAPTA-AM in serum-starved HEK293A cells; this partially reduced phosphorylation at both examined tyrosine residues. In

parallel, PI3K inhibition with wortmannin also partially decreased SHIP2 phosphorylation. Combining BAPTA-AM and wortmannin produced an additive reduction. Finally, inhibiting PLC with U73122 (but not the inactive analogue U73343) markedly lowered EGF-induced SHIP2 phosphorylation.

4.5. Evaluation of GRP1-PH and Akt-PH mutants for specific detection of PM PIP_3 dynamics

To obtain lipid-specific readouts of plasma-membrane PIP_3, we compared wild-type versus protein-interaction deficient mutant PH domains from GRP1 (K340L, I307E) and Akt (T34L, T34F) in a BRET assay. HEK293A cells were stimulated with EGF, insulin or sodium orthovanadate, and biosensor recruitment to the PM was quantified using BRET. Wild-type GRP1-PH showed strong responses to all stimuli, whereas K340L and I307E were markedly blunted, indicating that GRP1-PH recruitment depends substantially on protein interactions in addition to lipid binding. In contrast, Akt-PH mutants retained robust, stimulus-dependent recruitment with only modest amplitude loss versus wild type, suggesting Akt-PH readouts are less confounded by non-lipid interactions. Thus, GRP1-PH mutants improve specificity but at the cost of

sensitivity, while Akt-PH (including T34L/F) provides a more reliable PIP_3 reporter for dynamic PM signaling.

4.6. Improving the affinity and performance of mutant GRP1-PH-based biosensors through tandem domain engineering

To restore sensitivity lost in protein-interaction deficient GRP1-PH mutants, we created tandem mutant GRP1-PH versions to boost membrane avidity. In HEK293A cells BRET assays showed that the tandem mutants produced much larger stimulus-evoked signals to EGF, insulin and sodium orthovanadate, reaching amplitudes comparable to wild-type GRP1-PH. Confocal imaging of Venus-tagged constructs showed cytosolic and nuclear localization and, upon EGF, a rapid plasma-membrane translocation for the tandem sensors, demonstrating improved responsiveness and suitability for live-cell visualization of PIP_3 dynamics.

5. Conclusions

The major conclusions of our studies are as follows:

Investigation of the dynamics and regulation of $PI(3,4)P_2$ production in response to EGF and insulin signaling:

1. We found that $PI(3,4)P_2$ and PIP_3 exhibit temporally and quantitatively distinct dynamics at the plasma membrane in response to EGF and insulin. EGF preferentially promoted sustained $PI(3,4)P_2$ accumulation relative to PIP_3, highlighting receptor-specific phosphoinositide responses.
2. Our data confirmed SHIP2 as the major phosphatase responsible for $PI(3,4)P_2$ generation downstream of EGF, with minimal involvement following insulin stimulation. siRNA-mediated knockdown of SHIP2 reduced EGF-induced $PI(3,4)P_2$ accumulation, validating its enzymatic role.
3. We identified that EGF-induced SHIP2 phosphorylation is calcium-dependent and mediated through PLC signaling. This post-translational modification enhances SHIP2 phosphatase activity, contributing to increased $PI(3,4)P_2$ levels during EGF signaling.

Optimizing PH domain-based biosensors for improved detection of plasma membrane PIP_3:

1. Targeted mutations in the GRP1-PH domain significantly reduced protein-mediated membrane association, thereby enhancing lipid selectivity. However, these mutations also led to a substantial reduction in signal intensity, necessitating the development of tandem versions of the mutated constructs to restore signal strength and enable their practical application in live-cell assays. In contrast, Akt-PH mutants retained strong membrane responsiveness, indicating lower dependence on protein interactions.
2. The optimized tandem GRP1-PH constructs were successfully validated in both BRET-based assays and confocal imaging. These biosensors reliably tracked PIP_3 dynamics with improved specificity and versatility across experimental platforms.

6. Bibliography of the candidate's publications

Publications relevant to the dissertation:

1. **Damouni , Amir**; Tóth, Dániel J; , Barsi, Szilvia; Nagy, Dániel Károly; Kasbary, Alexander; Hunyady, László; Cserző, Miklós; Várnai, Péter. Differential activation of the inositol 5-phosphatase SHIP2 by EGF and insulin signaling pathways. Journal of Biological Chemistry, 2025, 301(7), 110275. IF(2024): 3.9
2. **Damouni, Amir**; Tóth, Dániel J.; Schönek, Aletta; Kasbary, Alexander; Boros, Adél P.; Várnai, Péter. Optimizing PH Domain-Based Biosensors for Improved Plasma Membrane PIP3 Measurements in Mammalian Cells. Cells, 2025, 14(14), 1125. IF(2024): 5.2

Cumulative impact factor: 9.1

Publications unrelated to the dissertation:

1. Tóth, Dániel J.; Tóth, József T.; **Damouni, Amir**; Hunyady, László; Várnai, Péter; Effect of hormone-induced plasma membrane phosphatidylinositol 4,5-bisphosphate depletion on receptor endocytosis suggests the importance of local regulation in phosphoinositide

signaling. Scientific Reports, 2024, 14, Article 291. IF (2024): 3.9

2. Gém, Janka Borbála; Kovács, Kinga Bernadett; Barsi, Szilvia; Hadadnejadtehrani, Sahar; **Damouni, Amir**; Turu, Gábor; Tóth, András Dávid; Várnai, Péter; Hunyady, László; Balla, András. Role of LMCD1 in the Long-Term Effects of Angiotensin II in Vascular Smooth Muscle Cells. International Journal of Molecular Sciences, 2025, 26(9), 4053. IF(2024): 4.9

Cumulative impact factor of all publications: 17.9

Cumulative impact factor of first author publications: 9.1