A Novel Mislocalization System for Identification of the Spatially Instructive Mediators of Eukaryotic Chemotaxis

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Abstract

Phosphoinositide 3-kinase and the Rho GTPases are molecules that play a major role in determining how a cell will respond to a gradient of chemoattractant. In this study a technique is explored to artificially mislocalize these molecules and to investigate their potential roles as spatially instructive mediators of polarity. This technique involves the recruitment of the target molecules to chimeric receptors on the cell membrane. Biotinylated antibodies and streptavidin-coated beads cluster receptors at a specific region. Significant progress was made in developing this method and in optimizing the experimental design. This novel technique may be used to analyze the function of these molecules, and it may be applied to a host of interactions in which spatial distribution is a significant component.

1 Introduction

An essential function of many eukaryotic cells is the ability to migrate in response to an extracellular gradient of chemoattractant ligand. This directed movement, known as chemotaxis, is necessary for proper operation of a broad array of biological processes ranging from the movement of neutrophils of the immune response to the passage of axonal projections through the embryonic nervous system. Bacterial cells interpret this gradient using a temporal strategy by comparing levels of ligand molecules over a given time period. Eukaryotic cells, however, adopt a spatial signaling system for gradient interpretation. Chemotaxis in eukaryotic cells is accomplished by comparing levels of chemoattractant across the cell surface and then adopting a polarized morphology, developing defined leading and trailing edges oriented with respect to the gradient [1]. Although cellular chemotaxis was identified over a century ago [2], a substantial portion of the reactions that direct movement remain poorly characterized. It has been conjectured that a spatially instructive mediator exists and directs cell polarization.

Thus far, two families of molecules have emerged as candidates for the spatially instructive mediator. The phosphoinositide 3-kinases (PI3-kinases) are lipid kinases that phosphorylate the 3-position of the inositol ring of phosphoinositides (PI) [3]. These kinases consist of a regulatory subunit, p85, and a catalytic subunit, p110. One product of this enzyme is the molecule phosphatidylinositol-3,4,5-P_3 (PIP_3), which has been shown to be not only the most upstream molecule in the cascade that is distributed asymmetrically but also the most downstream molecule able to induce polarization [4]. The second family, the Rho guanine triphosphatases (Rho-GTPases), is involved in a positive feedback loop to enhance PIP_3 production and accumulation [5]. This family includes the GTP-binding protein Rac, which is the primary GTPase investigated in this study. Rather than directly interfering with Rac for experimentation, the behavior of this GTPase is manipulated by controlling P-Rex, a

guanine nucleotide exchange factor (GEF). P-Rex serves to replace the guanine diphosphate in the inactive Rac with guanine triphosphate, re-activating the GTPase. PI3-kinase is also expressed in an inactive form and is activated by recruitment to the membrane.

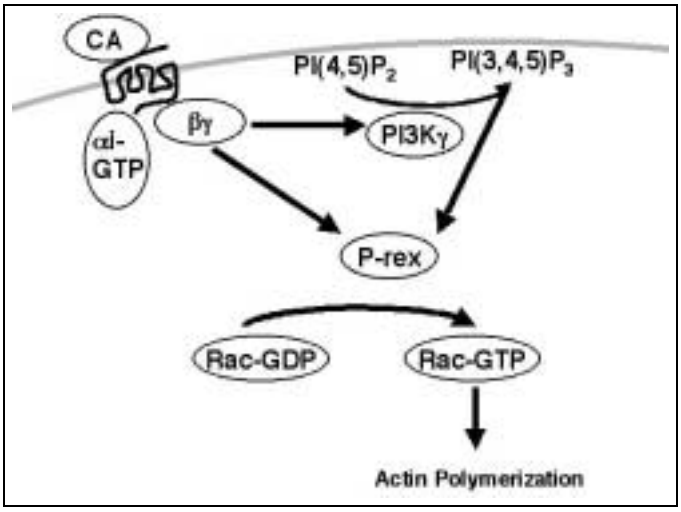

Figure 1: Signal transduction cascade for P-rex activation. Stimulation of a G-coupled receptor (membrane protein) by chemoattractant (CA) induces the activation of the heterotrimeric G-protein ($\alpha\beta\gamma$) and the dissociation of Gα from G$\beta\gamma$. G$\beta\gamma$ directly stimulates PI3-kinase, leading to the production of PIP_3. G$\beta\gamma$ and PIP_3 interact in a synergistic fashion to activate P-rex, which, in turn, catalyzes the exchange of GDP for GTP in the Rac molecule. The activated Rac subsequently stimulates the actin polymerization necessary for chemotaxis.

This study examines whether PI3-kinase or Rac is sufficient to direct cell polarization and motility. Although PIP_3 is known to induce cell polarity, the reactions that determine the direction in which the cell will move, particularly those occurring in a uniform concentration of chemoattractant, have yet to be identified. To accomplish this goal a novel technique has been devised that differs significantly from traditional knockout experiments. Instead of observing loss of function in negative mutants, each of the two possible effectors is artificially mislocalized and concentrated at a single point along the cell membrane. The advantage of this method is that it reflects the natural asymmetric properties of these molecules. Additionally, this process utilizes the endogenous materials of the cell rather

than introducing foreign proteins, providing an environment conducive to innate activity in a physiological context. The results of this study will not only enhance the understanding of these cellular mechanisms but will also aid the development of novel therapeutic agents to treat cancer metastases, neural degeneration, and other illness resulting from an aberrant interface between extracellular signals and the intracellular environment.

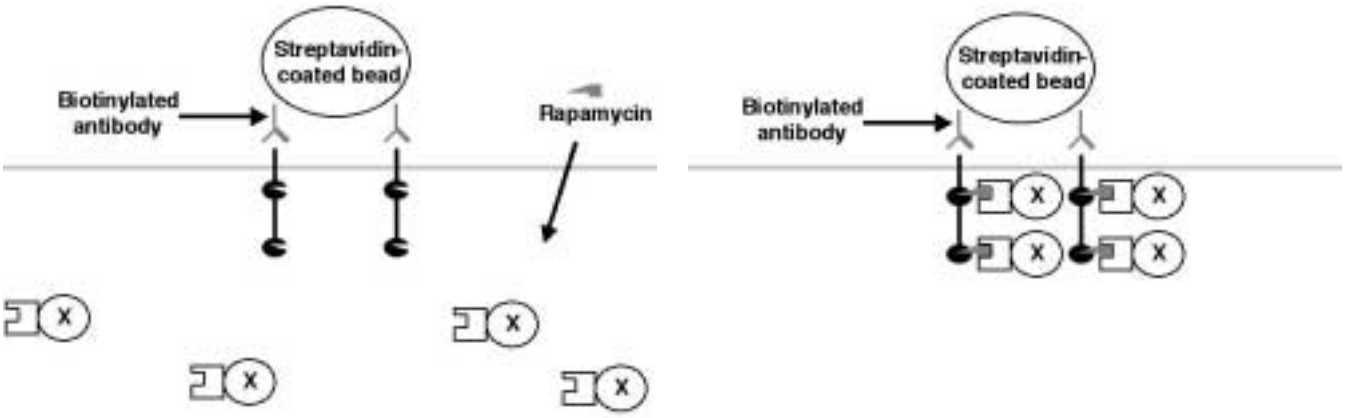

Figure 2: Rapamycin induces protein dimerization, linking the FKBP receptors with the FRAP-bound target molecule (designated with an X) Biotinylated antibodies bind to the the CD25 receptor on the extracellular face of the plasma membrane, and these antibodies are clustered using streptavidin-coated beads.

The experimental mislocalization system utilized in this study depends upon the presence of two basic components: a region to which target molecules migrate and a mechanism to transport these molecules. The first portion of this system was constructed by exploiting the ability of a specific cell-permeable immunosuppressant to induce protein dimerization [9]. This compound, known as rapamycin, binds with high affinity to a FK506-binding protein (FKBP). Rapamycin, in turn, recruits another protein known as FRAP (FKBP-rapamycin-associated protein), linking FKBP to FRAP. A chimeric construct, CD25-$FKBP_2$, was developed to fuse the extracellular and transmembrane regions of CD25 receptor protein to two

tandem copies of FKBP rapamycin-binding complex. On the extracellular face of the plasma membrane, antibodies against the exposed CD25 bind to the CD25-$FKBP_2$ membrane element. These antibodies are coated with a molecule known as biotin, a small, water-soluble B vitamin used in concert with streptavidin-coated beads. When placed adjacent to the cell, the streptavidin adheres tightly to the biotin and clusters the antibodies, concentrating the CD25-$FKBP_2$ complexes at a single site in the plasma membrane. The second component of this process involves transfecting cells to express a version of either PI3-kinase or P-Rex that is modified by attaching the active portion of FRAP. Consequently, upon exposure to rapamycin, the molecules are recruited to the plasma membrane, cluster at the CD25-$FKBP_2$ complex, and are effectively concentrated in a specific region.

2 Materials and Methods

The experimental system has been divided into two phases. Phase One has been successfully completed, and Phase Two is currently underway.

2.1 Phase One

Creation of CD25-$FKBP_2$ membrane element Rat CD25 DNA (nucleotides 1–1578, accession number P26897) was amplified with PCR, and primer sequences were designed to incorporate in-frame XbaI restriction sites. The products and the destination vector were digested with XbaI for 2 hours at 37°C and ligated into vector pC_4M-F2E (Ambian) with T4 DNA Ligase for 2 hours at room temperature according to the manufacturer's instructions (New England Biolabs). 1.5 μl of calf alkaline phosphatase (New England Biolabs) was added to the vector digestion reactions to phosphorylate the ends and prevent the vector from re-ligating. Reaction volumes for the ligation were as follows: 1 μl 10X T4 DNA Ligase buffer, 1 μl T4 DNA Ligase, 1 μl destination vector, and 7 μl target DNA. Water was used in place

of target DNA for the control ligations. The vector was amplified through transformation into chemically competent cells and was subsequently extracted from the ampicillin-resistant colonies. Vector samples were isolated and purified with agarose gel electrophoresis.

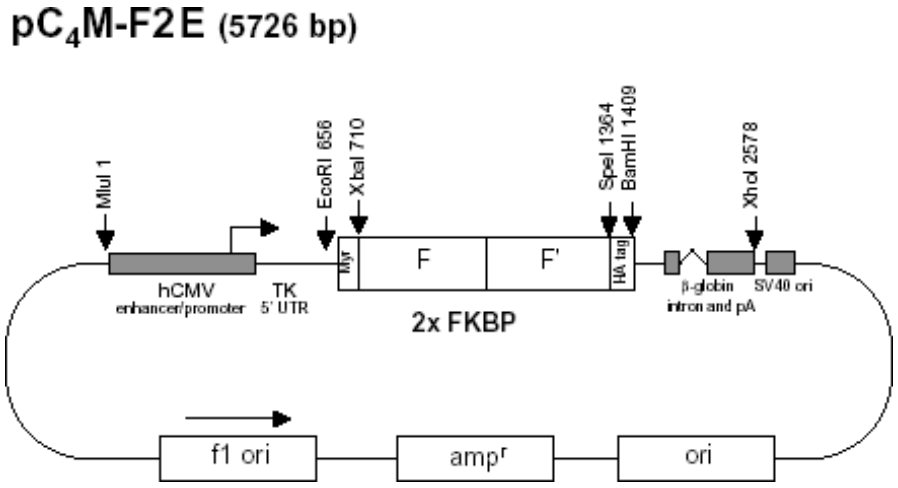

Figure 3: The pC_4M-F2E vector contains an amino-terminal myristoylation signal (M), two tandem copies of FKBP (F2), and a carboxy-terminal epitope tag (E, from the influenza hemagglutinin gene). One of the FKBP sequences has codon changes that, while not changing the amino acid sequence, are sufficiently different from the original sequence that the match between the two domains is dramatically reduced, thus decreasing the probability of recombination. The protein is expressed under control of the human CMV enhancer/promoter (C). Fusions of proteins to the FRB domain were accomplished by cloning the desired DNA into either the XbaI restriction site (N-terminal fusions) or the SpeI restriction site (C-terminal fusions).

Cell culture and transfections NIH 3T3 cell lines were propagated in DMEM (Dulbecco's Modification of Eagle's Medium, Mediatech), 10% fetal calf serum (Gemini Bio-Products), 50 units per mL penicillin (Invitrogen), and 50 units per mL streptomycin (Invitrogen). Transient transfections were accomplished using the FuGENE 6 Transfer Reagent system (Roche Diagnostics Corporation) according to the manufacturer's instructions with 2 μg of DNA. FuGENE, a non-liposomal transfection reagent, was selected for its minimal cytotoxicity and its function in serum-containing media.

Immunofluorescence Cells were trypsinized and re-plated on glass coverslips lined with silicon spacers (Nalge Nunc International). Cells were fixed in 3.7% formaldehyde and 11% sucrose in cytoskeleton buffer (10 mM PIPES pH 6.2, 138 mM KCl, 3 mM $MgCl_2$, and 2 mM EGTA pH 8) at room temperature for 20 minutes and permeabilized in 0.1% Triton-X100. Cells were stained with rhodamine-phalloidin to visualize the actin cytoskeleton. CD25-$FKBP_2$ immunostaining was achieved by adding 100 μl of a 1:100 biotinylated anti-CD25 antibody to 16–36 hour transfected cells. These cells were incubated at 37°C for 45 minutes and then stained with 100 μl of 1:100 TexasRed-Avidin in plain DMEM. The CD25-$FKBP_2$ construct could be visualized in the red channel. Cells were viewed using a Nikon Diaphot 300 Inverted Microscope using a Nomarski imaging technique.

Constructs The constructs utilized were pEGFP, p110, pEGFP-110, p85/p110, and P-Rex. pEGFP is green fluorescent protein, nucleotides 1–735 (accession number P42212), p110 is the p110α subunit of PI3-kinase, nucleotides 1–3204 (accession number P42337), and pEGFP-110 is a fusion of these two structures. p85/p110 is a heterodimer of mouse iSH_2 p85α domain, nucleotides 466–567 (accession number M60651) and mouse p110α, nucleotides 21–1061 (accession number U0327g). P-Rex is the GEF for Rac, nucleotides 1–4977 (accession number Q8TCU6). All constructs were amplified by PCR to create in-frame XbaI restriction sites for cloning into vector pC_4-$R_{H1}E$ (Ambian) for FRB N- and C-terminal fusions.

2.2 Phase Two

Secondary construct vectors Construct vectors for p85/p110, P-Rex, and pEGFP will be digested and inserted into pC_4-$R_{H1}E$ vectors using the same method as the CD25-$FKBP_2$ membrane element. p110 and pEGFP-110, however, were found to have XbaI restriction sites within their coding sequence. Either different restriction enzymes sites will be added to the construct DNA or the PCR products will be inserted into cloning vectors to undergo

partial digestion.

Recruitment and clustering of membrane proteins Association of the chimeric proteins will be investigated through immunofluorescence microscopy. Cells on coverslips will be incubated with 100 nM rapamycin (or DMSO as a control) for 10 hours, followed by incubation with 5 μg per mL of biotinylated mouse anti-CD25 antibody (clone B1.49.9, Immunotech) for 1 hour. The cells will be washed with DMEM, and 1 μm-diameter green-fluorescent streptavidin-labeled latex beads (Sigma) will be added. Cells will be centrifuged at 400 x g for 2 minutes and incubated on ice for 20 minutes. Excess beads will be removed and media warmed to 37°C will be added. Cells will be observed for various incubation times and fixed in 3.7% formaldehyde. Images will be constructed in successive 0.25-μm focal planes through the sample with interference light removed with a constrained iterative deconvolution algorithm [11, 12].

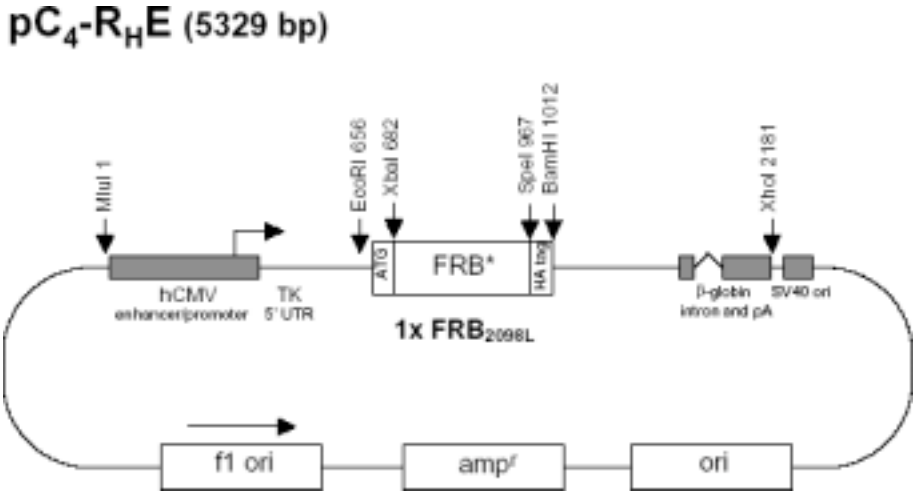

Figure 4: The pC$_4$-R$_{H1}$E vector is a chimeric fusion protein containing a single copy of FRB (R$_{H1}$) followed by a carboxy-terminal epitope tag (E, from the influenza hemagglutinin gene). Control of expression and fusion restriction sites are identical to those of the pC$_4$M-F2E vector, as described above.

3 Results and Data

CD25-FKBP$_2$ was successfully transfected into eukaryotic cells Restriction digests with extracted plasmid revealed which samples contained vectors with correct orientation of the CD25 coding sequence. These samples were utilized to achieve transfections with the CD25-FKPB$_2$ chimera. Both FuGENE and Lipofectamine Plus transfer reagents were tested to determine which would be more effective, and for each reagent multiple concentrations of target DNA were explored. Lipofectamine Plus resulted in a lower yield of positive clones and severely damaged a significant percentage of the cells. FuGENE, a non-liposomal transfer reagent, was found to have a significantly lower degree of cytotoxicity and was thus used for the remainder of the transfections.

Treatment with antibody produces primary clustering of CD25 receptors Immunostaining with anti-CD25 antibody aggregated the membrane receptors at multiple positions along the cytoplasmic membrane. These clusters, appearing as bright foci under green excitation, were clearly visible under 60x magnification and appeared to be evenly distributed across the face of the plasma membrane.

pEGFP, p110, pEGFP-110, p85/p110, and P-Rex constructs were correctly amplified using PCR Each of these constructs was amplified using PCR and optimized for various elongation times and temperatures. Agarose gel electrophoresis indicated that products of the correct length were produced. However, when these products were digested with XbaI for ligation into a vector, the p110 and pEGFP-110 constructs were cleaved into 2 segments, indicated an additional, unforeseen restriction site within the original DNA construct. Further investigation revealed that mislabeling had occurred during the shipping process and that the p110 used was of a different strain than originally indicated. Future plans for these two constructs involve running partial digests and selection of correctly cleaved segments or

possibly site-directed mutagenesis.

Progress with secondary vectors Preliminary data indicate that p85/p110 was inserted into a vector in the proper orientation. Transformations with these plasmid samples produced experimental plates with a 100-fold increase in colonies when compared with the control plates, which were transformed with the vector-only ligation. Extraction and purification of the vectors from the cell cultures is currently underway.

4 Discussion

PI3-kinase and the Rac GTPase are both potential mediators for directing cell polarity. Classical methods to elucidate biochemical pathways involve observing the effects of either a gain or loss of function of the molecule in question. Although this approach has the advantage of simplifying experimental design, it may introduce abnormalities in both the spatial and the temporal regulation of these molecules. To truly understand the processes of cell polarization of chemotaxis, the spatial distribution of the molecules must be considered, with activity analyzed in a localized manner. In this study a technique of protein dimerization was developed to induce artificial mislocalization of the molecules undergoing investigation. This technique circumvents the problem of uniform insertion by inserting coding sequences into the cells and recruiting the expressed molecules to the membrane. Additionally, by introducing the molecules in an inactive form, spontaneous activation may be induced to mimic the natural reactions of intracellular signaling.

A variety of difficulties were initially encountered in the implementation of the procedure. As a result of a simple shipment error, constructs p110 and pEGFP-110 exhibited XbaI restriction sites within the coding sequence. Consequently, XbaI digestion cleaved these sequences in half and prevented their insertion into a vector. Possible solutions to this

problem include site-directed mutagenesis, the creation of new construct DNA, and insertion into a cloning vector for partial digestion. However, even if these two constructs are transfected into eukaryotic cells, it is probable that the proteins will display erratic behavior. These constructs contain only the catalytic subunit of PI3-kinase, and the molecule is relatively unstable when detached from its regulatory subunit. p85/p110 was thus designed and is expected to function properly, as p110 is bound to the stabilizing domain (iSH_2) of p85. Additional difficulties were experienced in distinguishing colonies that had taken up the plasmid with the insert from the background of colonies transformed with the re-ligated vector. Initial results indicate that this obstacle may be overcome by performing the ligation reactions at 16°C. This observation is reasonable, since the T4 DNA Ligase enzyme degrades much more slowly at lower temperatures and is, as a result, able to phosphorylate the vector sample more thoroughly.

The introduction of new constructs into eukaryotic cells raises the issue of potential interference from endogenous signaling molecules. The vectors that are inserted into the cells are equipped with a strong viral promoter from human CMV. Use of this promoter results in the expression of large amounts of the modified proteins, which are either inactive or accessory activators. Recruitment to the membrane receptors activates these molecules. FKBP and FRAP segments are sufficiently distinct from receptors present in the transfected cells to prevent unintended interactions. Furthermore, the molecules of the cell are distributed evenly prior to polarization and would not bias the activity of the experimental system.

For each of the hypothetical spatial mediators, outcomes are expected to fall into one of two main categories. The first possibility is that cell polarity is uncorrelated with the localization of the recruited molecules. This phenotype would indicate either that the molecules have a permissive role in the determination of cell polarity or that they require additional effectors to direct intracellular reactions. The second possibility is that cell polarization is aligned with the localized molecules, implicating the molecule as either sufficient to direct

cell polarity or capable of biasing polarity to a significant degree. The role of these molecules could be further elucidated by analyzing various stages of polarity in a uniform gradient of chemoattractant. One observation may be that multiple leading edges form, indicating that the molecule plays a role in directing cell polarity but is incapable of communicating with the entire cellular cascade. An alternate observation may be the formation of leading edges aligned with the recruited molecules, with additional leading edge formation repressed. This result would imply that the molecule is not only capable of inducing polarization but also able to regulate the other activities of the cell.

As with any sufficiently new technology, a great deal of time must be invested into optimization of techniques and adaptation of procedures. Through this study much progress has been achieved in the area of artificial mislocalizations, and this technique may be applied to a host of other experimental systems.

5 Conclusion

A novel technique was explored to investigate the role of PI3-kinase and Rac GTPase in the organization of cell polarity. This method may be utilized not only for examining these specific molecules but also for studying a variety of other spatially relevant processes. Increased development of technologies for both spatially and temporally manipulating intracellular signaling are essential to unravel the complex interactions underlying cellular chemotaxis.

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References

[1] Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. (1994) *Molecular Biology of the Cell (Third Edition).* New York: Garland Publishing.

[2] Berg, H. C. (1988) A physicist looks at bacterial chemotaxis. *Cold Spring Harbor Symposium on Quantitative Biology,* **53:** 1–9.

[3] Weiner, O. D., Servant. G., Parent, C. A., Devreotes, P. N., and Bourne, H. R. Cell polarity in response to chemoattractants. In *Cell Polarity: Frontiers in Molecular Biology.* New York: Oxford University Press.

[4] Weiner, O. D., Neilsen, P. O., Prestwich, G. D., Kirschner, M. W., Cantley, L. C., and Bourne, H. R. (2002) A $PtdInsP_{3-}$ and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity. *Nature Cell Biology,* **1:** 75–81.

[5] Bourne, H. and Weiner, O. (2002) Directed polarity and motility: how does the compass work? *Nature,* in press.

[6] Heldin, C. H. Signal transduction: multiple pathways, multiple options for therapy. *Stem Cells,* **19:** 295–303.

[7] Rameh, L. E. and Cantley, L. C. (1999) The role of phosphoinositol 3-kinase lipid products in cell function. *The Journal of Biological Chemistry,* **274:** 8347–8350.

[8] Weiner, O. D. (2002) Regulation of cell polarity during eukaryotic chemotaxis: the chemotactic compass. *Current Opinion in Cell Biology,* **14:** 196–202.

[9] Castellano, F., Montcourrier, P., Guillemot, J., Gouin, E., Machesky, L., Cossart, P., and Chavrier, P. (1999) Inducible recruitment of Cdc42 or WASP to a cell-surface receptor triggers actin polymerization and filopodium formation. *Current Biology,* **9:** 351–360.

[10] Servant, G., Weiner, O., Herzmark, P., Balla, T., Sedat, J., and Bourne, H. (2002) Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science,* **287:** 1037-1040.

[11] Adard, D., Hiraoka, Y., Shaw, P., and Sedat, J. (1989) Fluorescence microscopy in three dimensions. *Seminars in Cell Biology.* **30:** 353–377.

[12] Swedlow, J., Sedat, J., and Agard, D. (1997) Deconvolution in optical microscopy. In *Deconvolution of Images and Spectra.* San Diego: Academic Press.

[13] Weiner, O. (2002) Rac Activation: P-rex1 - A convergence point for PIP_3 and $G\beta\gamma$? *Current Biology,* **12:** R429-R431.