

MEASURING PHOSPHORYLATED AKT AND OTHER PHOSPHOINOSITIDE 3-KINASE-REGULATED PHOSPHOPROTEINS IN PRIMARY LYMPHOCYTES

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Abstract

Phosphoinositide 3-kinase (PI3K) is a lipid kinase whose activation is crucial for many biological functions in multiple cell types. One research area of particular interest for basic biologists and drug developers is PI3K signaling in lymphocytes. Inhibitor studies and PI3K mutants have demonstrated that PI3K is required for development, activation, proliferation, differentiation, and survival of B lymphocytes, as well as optimal activation and proliferation of T lymphocytes. As the actual products of PI3K can be difficult to measure, the field has often adopted the practice of examining the activation of downstream effectors of PI3K, with the most common readout being phosphorylation of Akt. This chapter discusses key pathways influenced by PI3K signaling and the advantages and caveats of using activation of these pathways as indicators of PI3K activity. In addition, we provide traditional immunoblotting methods of assaying PI3K-dependent pathway activation, as well as more recent flow cytometry-based approaches (termed “phosflow”). Although we describe assays optimized for B lymphocytes, these methods are easily adapted to T lymphocytes and other leukocyte cell types.

1. OVERVIEW

1.1. Phosphoinositide 3-kinase (PI3K) introduction

The PI3K family of lipid kinases phosphorylates the 3-hydroxyl position on the inositol head group of phosphatidylinositol (PtdIns) lipid species embedded in the plasma membrane. There are multiple classes of PI3K, defined primarily by mode of activation and substrate selectivity (for detailed reviews, see [Deane and Fruman, 2004](#); [Vanhaesebroeck *et al.*, 2001](#)). Class IA and class IB PI3K are the only classes able to generate the second-messenger phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃]. Cellular proteins containing modular domains that bind PtdIns(3,4,5)P₃ are recruited to the plasma membrane following class I PI3K activation. Chemical inhibitors of PI3K (wortmannin or LY294002) block murine B- and T-cell activation and proliferation, demonstrating the importance of this kinase to lymphocyte function. Class IA PI3K is generally activated downstream of tyrosine kinase receptors in lymphocytes, whereas class IB PI3K is activated downstream of G-protein-coupled receptors. Both class IA and IB PI3Ks exist as heterodimers of a catalytic and a regulatory subunit. There are now numerous mouse strains with targeted deletion of one or more class I PI3K catalytic or regulatory subunit ([Deane and Fruman, 2004](#); [Fruman, 2007](#)). Most of these have interesting immune phenotypes; however, much work remains to be done to understand the impact of these knockouts on PI3K signaling mediated by diverse receptors on lymphocytes. One purpose of this chapter is to provide standardized protocols for assessing PI3K-signaling

output in lymphocytes lacking PI3K family members or treated with isoform-selective inhibitors that are emerging from pharmaceutical company screens (Barber *et al.*, 2005; Bilancio *et al.*, 2006).

1.2. PI3K signaling in B lymphocytes

Phosphoinositide 3-kinase is activated in B lymphocytes downstream of multiple receptors, including the B-cell antigen receptor (BCR), CD40, Toll-like receptors (TLRs), and numerous cytokine receptors (Fig. 8.1). In B-cell lines such as WEHI-231 or A20, BCR-mediated PI3K activation is characterized by transient increases in PtdIns(3,4,5)P₃ levels in the membrane, usually accompanied by a delayed increase in phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P₂] levels (Astoul *et al.*, 1999; Gold and Aebersold, 1994). For quantitation of PI3K kinase activity, the levels of these two PtdIns species can be measured directly via thin-layer

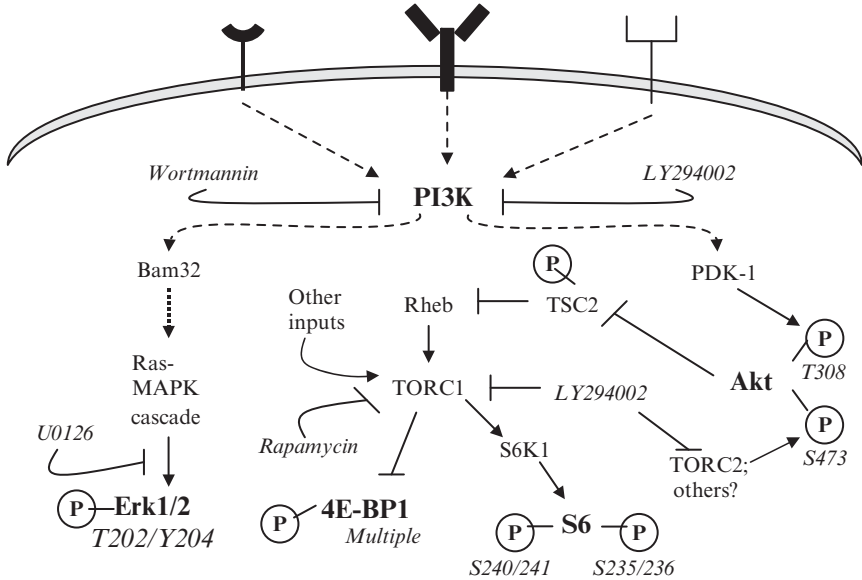


Figure 8.1 Readouts of PI3K activity. PI3K is activated downstream of many receptors in lymphocytes, including the antigen receptor, CD40, TLRs, and multiple cytokines. PI3K activity, through production of 3-phosphorylated phosphoinositides (not shown), leads in turn to the activation of other pathways and the phosphorylation of targets that can be used as readouts of PI3K activation, as described in the text. Protein names in bold represent downstream readouts discussed herein; italicized residues are phosphorylation sites analyzed. Note that S6 residues S240/241 are shown; these sites are PI3K dependent in some systems and can be assayed by immunoblot but not phosflow in our hands. Hatched arrows represent unknown intermediates or steps omitted for simplicity. “Other inputs” leading to activation of the TORC1 (mTOR-Raptor) complex include serum components, amino acids, and glucose, as described in the text.

chromatography (TLC) or high-performance liquid chromatography (see [Guillou *et al.*, 2007](#)). However, these assays require specialized equipment and large amounts of radioactivity. Moreover, labeling of primary lymphocytes requires a prohibitively large cell number for adequate detection and is complicated by the rapid death of these cells *ex vivo*. Other techniques for direct measurement of PtdIns(3,4,5)P₃ have been described but these methods have questionable sensitivity and/or specificity when applied to primary lymphocytes (see [Section 5](#)). These considerations have led to a search for alternative approaches for ascertaining whether PI3K has been activated. Engagement of each of the receptors mentioned earlier activates a unique suite of pathways. However, many of the downstream effectors of PI3K are conserved between these pathways. For this reason, it is possible to make use of common PI3K targets as readouts of its activation. Although these approaches provide only an indirect measurement of PI3K-signaling output, quantitative data can be obtained and, in many contexts, the results are quite informative. The following sections discuss various PI3K-regulated phosphoproteins before presenting specific protocols for measuring their phosphorylation.



2. CHOOSING A DOWNSTREAM READOUT: GENERAL CONSIDERATIONS

2.1. Downstream readout: Akt phosphorylation

The most established PI3K target in many systems is the serine/threonine kinase Akt ([Deane and Fruman, 2004](#); [Fruman, 2004](#)). With an ever-increasing number of known substrates, Akt appears to be crucial for survival and proliferation of B cells following BCR engagement ([Yusuf *et al.*, 2004](#)) and is involved in signaling following stimulation through other receptors as well. Activation of Akt requires phosphorylation of two important sites: threonine 308 (T308) in the activation loop and serine 473 (S473) ([Fig. 8.1](#)). T308 is phosphorylated by PDK-1 ([Alessi *et al.*, 1997](#); [Stephens *et al.*, 1998](#)), whereas several kinases might mediate phosphorylation of S473. One likely S473-kinase is the mammalian target of rapamycin (mTOR) complex 2 (TORC2; see later) ([Sarbasov *et al.*, 2005](#)). Both of these phosphorylation events appear to be PI3K dependent, as there is little or no phosphorylation at these sites in unstimulated cells, and mitogen-stimulated Akt phosphorylation is blocked by pharmacological inhibitors of PI3K and reduced in lymphocytes from various PI3K knockout mouse strains ([Bilancio *et al.*, 2006](#); [Clayton *et al.*, 2002](#); [Deane *et al.*, 2007](#); [Glassford *et al.*, 2005](#); [Hess *et al.*, 2004](#); [Okkenhaug *et al.*, 2002](#); [Suzuki *et al.*, 2003](#); [Vigorito *et al.*, 2004](#)). Furthermore, Akt phosphorylation is observed downstream of receptors that activate either class IA PI3K

(e.g., antigen receptors, CD40, cytokine receptors, TLRs) or class IB (e.g., chemokine receptors) (Andjelic *et al.*, 2000; Guo and Rothstein, 2005; Ortolano *et al.*, 2006; Venkataraman *et al.*, 1999; Vivarelli *et al.*, 2004). For these reasons, the degree of phosphorylation of Akt is an excellent correlate of PI3K activity in cells. As emphasized later, samples treated with global PI3K inhibitors such as wortmannin are useful specificity controls by revealing any PI3K-independent “background” signal in various types of assays. It is important to stress that activation of Akt does not mean that all PI3K-dependent responses will be activated to a similar extent. For example, PI3K-dependent Ca^{2+} mobilization in B cells occurs downstream of the BCR and chemokine receptors, but not via CD40 or most cytokine receptors that nevertheless trigger Akt phosphorylation.

2.2. Downstream readout: mTOR activation

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that functions in one of two complexes: the rapamycin-sensitive mTOR complex-1 (TORC1) containing the adaptor Raptor and the rapamycin-insensitive mTOR complex-2 containing the adaptor Rictor (TORC2) (Fig. 8.1). These complexes exhibit differential regulation and are responsible for the phosphorylation of different substrates. Activation of mTOR in lymphocytes is crucial for mediating the protein synthesis and cell size increases required for proliferation (Richardson *et al.*, 2004). Indeed, the drug rapamycin was first studied as an immunosuppressant and has been in clinical use for this purpose for many years. The best known substrates of TORC1 are 4E-BP1 and S6K, with S6K activation by mTOR leading to phosphorylation of the ribosomal protein S6. S6K and/or S6 is phosphorylated in primary B cells following stimulation with several mitogens, including cross linking anti-IgM antibodies (Abs), lipopolysaccharide (LPS), and anti-CD40+IL-4 (Bilancio *et al.*, 2006; Donahue and Fruman, 2003; Hess *et al.*, 2004). PI3K-dependent activation of TORC1 is mediated by Akt through phosphorylation of the tuberous sclerosis complex (TSC2) (Manning *et al.*, 2002). TSC2 is a negative regulator of TORC1 function via its negative regulation of the Rheb GTPase and is rendered inactive by phosphorylation on Akt-dependent sites (Richardson *et al.*, 2004; Tee *et al.*, 2003; Zhang *et al.*, 2003).

TORC1 activation occurs somewhat downstream from PI3K and is not as direct a readout as Akt phosphorylation (Fig. 8.1). TORC1 is also regulated by factors in serum, and TORC1 activity generally requires the presence of nutrients, most importantly amino acids and glucose (Kam and Exton, 2004; Wullschlegel *et al.*, 2006). The role of PI3K in these signaling inputs to TORC1 is not entirely clear and appears to vary among cell contexts. For example, in some B-cell tumor lines, TORC1 activity maintained by nutrients appears entirely independent of PI3K and occurs in the

absence of Akt phosphorylation (Wlodarski *et al.*, 2005). It is also important to consider that nutrient-dependent TORC1 activity requires the wortmannin-sensitive class III PI3K enzyme in some cellular systems, not class I PI3K (Byfield *et al.*, 2005; Nobukuni *et al.*, 2005). BCR-mediated phosphorylation of S6K, S6, and 4E-BP1 in splenic B cells is substantially blocked by wortmannin and by a selective class IA inhibitor (Bilancio *et al.*, 2006; Donahue and Fruman, 2007), indicating that class IA PI3K signaling plays a major role in this particular pathway. However, inhibition of TORC1 signaling by PI3K inhibitors is rarely as complete as that seen with direct mTOR inhibition by rapamycin. With some stimuli, for example, LPS, TORC1 activation in B cells appears largely PI3K independent (Donahue and Fruman, 2007). The caveat that TORC1 activation is often not completely dependent on PI3K activation is important to remember when interpreting data involving mTOR signaling and emphasizes the importance of including parallel samples treated with rapamycin (total inhibition) and wortmannin (to define the PI3K-dependent component). However, as excellent antibodies (Abs) exist for detection of S6K, S6, or 4E-BP1 phosphorylation, this pathway can be readily used as a reliable readout of PI3K activity in systems where PI3K is known to contribute to TORC1 activation.

A final word of caution for mTOR analyses relates to the PI3K inhibitor LY294002. Like wortmannin, this compound blocks enzyme activity of most PI3K catalytic isoforms. However, LY294002 is a direct inhibitor of both TOR complexes at concentrations normally used to inhibit PI3K (Brunn *et al.*, 1996; Knight *et al.*, 2006). Indeed, LY294002-treated B cells have considerably lower TORC1 signaling than cells treated with wortmannin or a class IA inhibitor (Bilancio *et al.*, 2006; Wlodarski *et al.*, 2005). At higher concentrations (*i.e.*, 100 nM), wortmannin also can inhibit TOR kinase activity directly. Therefore, LY294002 should be avoided in studies of PI3K-dependent mTOR signaling, and wortmannin should be used at concentrations below 100 nM.

2.3. Downstream readout: Phosphorylation of Erk

Phosphoinositide 3-kinase is also involved in the activation of the Erk MAP kinase cascade in B cells through a pathway that may involve the PtdIns(3,4)P₂-binding protein Bam32 and/or PI3K-dependent Ras activation (Han *et al.*, 2003; Jacob *et al.*, 2002). Ras-mediated Raf and Mek activation downstream of the BCR leads to phosphorylation of Erk1/2, and this phosphorylation is mostly blocked by pretreatment of unfractionated B cells with PI3K inhibitors (Bilancio *et al.*, 2006; Hess *et al.*, 2004; Jacob *et al.*, 2002). Good antibodies also exist for phosphorylated Erk1/2, making this pathway another reliable readout of PI3K in some systems. Appropriate controls include samples treated with Mek inhibitors (which generally give

total inhibition of pErk) and samples treated with wortmannin (to define the PI3K-dependent fraction of the signal). However, Erk phosphorylation is not triggered (or is delayed) downstream of many receptors that mediate acute activation of Akt or mTOR. This stresses the importance of choosing relevant downstream readouts in a particular system.

3. PROTOCOLS FOR DETECTION OF PI3K-REGULATED PHOSPHOPROTEINS BY IMMUNOBLOT

For the pathways discussed earlier, reliable immunoblotting protocols have been used by many investigators to examine the phosphorylation state of PI3K targets. Phospho-specific Abs for Akt (S473 or T308), Erk1/2 (T202/Y204), and S6 (S235/236) detect these proteins only when they are phosphorylated on the indicated sites. Although some laboratories have used phospho-specific antibodies to S6 kinase 1 (S6K1) and 4E-BP1 (Bilancio *et al.*, 2006; Prabhu *et al.*, 2007; Wlodarski *et al.*, 2005), we have had more success detecting phosphorylation-dependent mobility shifts of these proteins during SDS-PAGE separation that can be detected by immunoblot with an Ab directed against total protein (Donahue and Fruman, 2003, 2007). The degree to which these phosphorylation events depend on PI3K activation can be assessed by incubation of a sample of cells with PI3K pharmacological inhibitors prior to stimulation. The immunoblot approach is most informative in cases where cell populations are homogeneous and abundant, such as lymphoma cell lines. When using primary lymphocytes, it can be difficult to obtain a sufficient number of cells to achieve a good signal-to-noise ratio, especially if the experiment calls for many conditions to be tested. In addition, heterogeneity in signaling responses among cell subtypes in mixed populations will be indistinguishable. This masking of heterogeneous responses, as well as the sometimes prohibitively large number of cells required for immunoblotting, is addressed in our discussion of flow cytometry-based assays.

3.1. Choice of inhibitors

Inhibitors are becoming available that will allow researchers to study the requirement for specific PI3K catalytic isoforms. For the purposes of this chapter, however, we discuss the standard pan-catalytic PI3K inhibitors LY294002 and wortmannin and the mTOR inhibitor rapamycin. LY294002, originally identified by the Lilly Corporation, binds reversibly to PI3K and is stable in culture and continues to inhibit over several days. In contrast, the naturally occurring compound wortmannin binds irreversibly but has a chemical half-life in culture of roughly 2 h. Both compounds are

light sensitive and can have off-target effects at higher concentrations. LY294002 inhibits the kinase activity of mTOR at a similar concentration to what is used for PI3K inhibition and must therefore be used with care and not in experiments examining mTOR targets. Although wortmannin can also have off-target effects, there appear to be effective concentrations at which the inhibitor is PI3K selective (i.e., ≤ 100 nM).

1. LY294002 and rapamycin should be diluted in 100% ethanol to a stock concentration of 10 mM, kept sterile and light protected, and stored at -80° in a vial with an O-ring cap to prevent evaporation of the solvent. Incubate cells at 5 to 10 μ M (for LY294002) or 10 nM (rapamycin) for 15 min at 37° prior to stimulation and compare with cells treated with a similar dilution of ethanol vehicle. An intermediate dilution in culture medium is usually necessary to achieve an accurate working concentration of rapamycin. It is important to note that rapamycin should not be used as a negative control inhibitor for other PI3K downstream readouts (e.g., pAkt), as mTOR inhibition can affect these pathways indirectly (Wullschleger *et al.*, 2006).
2. Wortmannin should be diluted in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mM. As repeated freeze/thaw cycles decrease the efficacy of the inhibitor, single-use aliquots of 2 to 3 μ l should be stored in light-protected tubes at -80° . An intermediate dilution in culture medium is usually necessary to achieve an accurate working concentration. Incubate cells at 25 to 100 nM for at least 15 min at 37° prior to stimulation and compare with cells treated with a similar dilution of DMSO vehicle.

3.2. Stimulation of primary B lymphocytes

We have found that detection of the phosphorylated proteins described herein requires at least 1.5 to 2×10^6 primary B lymphocytes per sample. If possible, 5×10^6 cells should be used for detection of pAkt (also for analysis of primary T cells). Purified B cells are obtained from murine spleen by magnetic separation as described previously (Donahue and Fruman, 2003). When using cell lines such as A20, detection is feasible using 0.5 to 1×10^6 cells. Cells should be at a concentration of 1×10^6 /ml and mixed with an equal volume of a warmed (37°) solution containing $2 \times$ final concentration of stimulus for a final concentration of 5×10^5 cells/ml. Note that the final concentration of any drugs present during the pretreatment period is also reduced by half; this is more relevant for reversible inhibitors such as LY294002 than for the covalent irreversible inhibitor wortmannin. Cell stimulations may take place in tissue culture plates in a 37° incubator or in microcentrifuge tubes in a 37° water bath. We find that although the use of microcentrifuge tubes requires multiple tubes for each sample, this approach

makes for a more efficient harvest of cells and therefore causes less variation in stimulation time from sample to sample, especially when treating for very short time periods (e.g., 1 min).

3.3. Harvest, lysis, and SDS-PAGE

Standardized procedures for cell harvest, lysis, SDS-PAGE, and immunoblot are available from many sources, including [Coico \(2006\)](#). Many investigators have used such methods or slight variations to detect PI3K-regulated phosphoproteins. The following sections present our own protocol while highlighting some important considerations for working with primary lymphocytes and phosphoproteins.

Pellet cells for 5 min at 160g (about 1100 rpm in a typical table-top centrifuge) for 5 min and wash twice with cold phosphate-buffered saline (PBS) to remove abundant serum proteins (i.e., albumin) and excess stimulus. If necessary, combine cells from each samples' multiple tubes during this washing process. Aspirate the supernatant following the final wash, disperse the cell pellet by flicking vigorously, and either snap-freeze cells and store at -20° or continue directly. Lyse with Triton X-100 lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol) containing protease and phosphatase I and II inhibitor cocktails (Sigma) at 1:100 dilution. Vortex and incubate lysates on ice for 10 min, vortexing roughly every 5 min. Spin lysates for 5 min at top speed in a microcentrifuge at 4° to pellet nuclei, and carefully transfer supernatants to fresh tubes. Add an equal volume of 2 \times SDS-PAGE sample buffer and heat lysates for at least 5 min at 95° . Lysates can then be stored frozen or immediately loaded onto an SDS-PAGE gel. If determination of protein concentration is desired, remove a small volume of the lysates prior to addition of the 2 \times sample buffer.

We find that the phosphoproteins described herein are of sufficiently different size (pAkt \approx 60 kDa; pErk \approx 42/44 kDa; pS6 \approx 32 kDa; 4E-BP1 \approx 15–20 kDa) that when resolved on a large 12% SDS-PAGE gel, their separation makes detection of all of them possible in a single immunoblot. Transfer proteins onto a filter support (standard nitrocellulose is adequate in our experience) and stain with Ponceau S to visualize protein and aid in cutting the membrane into sections, each containing the region around one protein of interest, for immunoblotting.

3.4. Immunoblotting

All of the primary Abs recommended here are obtained from Cell Signaling Technology and are detected using the same immunoblotting protocol: pAkt (S473) rabbit monoclonal Ab (mAb) or pAkt (T308) rabbit mAb, pErk1/2 or p44/p42 (T202/Y204) rabbit mAb, pS6 (S235/236) rabbit polyclonal Ab or mAb, and total 4E-BP1 rabbit polyclonal Ab. These and other key reagents described in this chapter are listed in [Table 8.1](#).

Table 8.1 Reagents and suppliers

Reagent	Supplier
pAkt (S473) rabbit mAb	Cell Signaling Technologies
pAkt (T308) rabbit mAb	Cell Signaling Technologies
pErk1/2 (T202/Y204) rabbit mAb	Cell Signaling Technologies
pS6 (S235/236) rabbit polyclonal Ab	Cell Signaling Technologies
pS6 (S235/236) rabbit mAb	Cell Signaling Technologies
4E-BP1 rabbit polyclonal Ab	Cell Signaling Technologies
Goat anti-rabbit IgG-A488 secondary Ab	Invitrogen (Molecular Probes)
Goat anti-rabbit IgG-FITC secondary Ab	Sigma-Aldrich
Goat anti-rabbit IgG-HRP	Promega
Goat anti-mouse IgG-HRP	Promega
CytoFix/CytoPerm kit	BD Biosciences
Methanol-free formaldehyde (16%)	Polysciences Inc
SuperSignal West Pico chemiluminescent substrate kit	Pierce
Restore Western blot stripping buffer	Pierce

Rock the membrane in blocking solution (5% nonfat dry milk in TBST: 100 mM Tris, pH 8.0, 500 mM NaCl, 0.1% Tween 20) for at least 1 h at room temperature or for longer periods at 4°. Despite reports that milk contains phosphatases that diminish the signal in antiphosphoprotein immunoblots, this has not been our experience for the target proteins described herein, and milk is in fact the blocking solution recommended by Cell Signaling. Then incubate the membrane with primary Ab at a 1:1000 dilution in a 5% bovine serum albumin (BSA)/TBST solution overnight at 4° on a nutator. Wash the membrane with TBST four times, 5 min each, and then add secondary Ab. Horseradish peroxidase-conjugated goat antirabbit IgG secondary Ab (Promega) should be used at a dilution of 1:10,000 in TBST and incubated for 1 h at room temperature on a shaker. Wash four more times with TBST, 5 min each. Visualize bound Ab by enzyme-linked chemiluminescence (ECL; Pierce SuperSignal West Pico chemiluminescence kit) and expose to film.

Equivalent protein loading can be demonstrated by probing the appropriate stripped and reblocked membrane section with primary Ab directed against total Akt protein (Cell Signaling, rabbit polyclonal Ab) or total S6 protein (Cell Signaling, rabbit or mouse mAbs). Antibodies to other proteins (e.g., β -actin; Sigma) can be used as loading controls. However, care should be used in titrating the primary antibody in order to achieve detection in the linear range of the film used for chemiluminescent detection. If the signal is too strong, a shortened secondary Ab incubation (e.g., 30 min) or a twofold dilution of the ECL solution can help. Membranes can

be stripped quickly and easily with Restore Western blot stripping buffer (Pierce) per the accompanying instructions.

3.5. Interpretation

A typical result for cytoplasmic extracts from purified murine B cells stimulated via BCR cross linking is shown in Fig. 8.2. In the case of the phospho-specific Abs (pAkt, pS6, and pErk1/2), stimulation through the BCR causes a robust increase in band intensity, representing phosphorylation at the targeted site. That these phosphorylation events are dependent on PI3K is demonstrated by the loss of signal in lanes containing lysates of cells pretreated with wortmannin. A low level of signal is sometimes detected in unstimulated cells and can be influenced by incubation time and composition of the media (Donahue and Fruman, 2007). It is helpful to remember that PI3K is activated by diverse extracellular stimuli and it is important to determine the basal level of signaling in a particular experimental system. Of note, the general conditions described here are also sufficient for detecting phosphorylation of Akt substrates, including TSC2 and FOXO proteins, using commercially available antibodies selective for Akt phosphorylation sites (Hess *et al.*, 2004; Yusuf *et al.*, 2004).

The Ab used for detection of 4E-BP1 here recognizes total protein, and interpretation of phosphorylation levels is dependent on migration of the

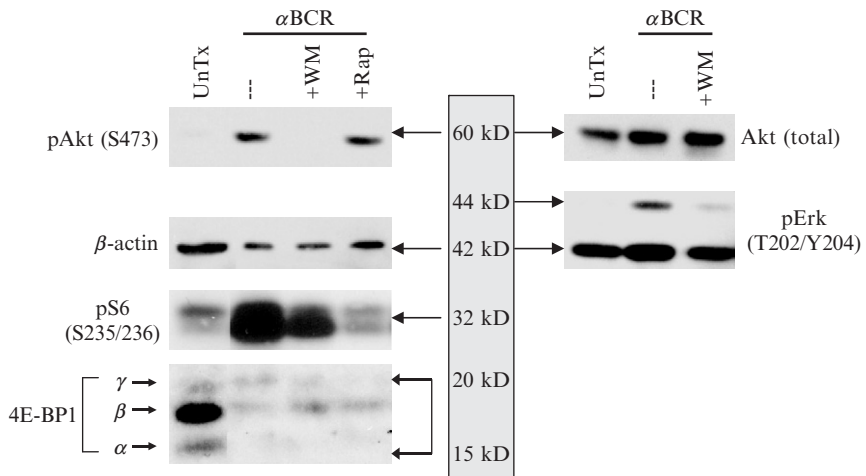


Figure 8.2 Immunoblot detection of PI3K-dependent phosphorylation events. Following BCR cross linking in purified primary B lymphocytes, phosphorylation of multiple PI3K readouts can be visualized on a single immunoblot. Representative experiments using 1.5 to 2×10^6 B cells resolved on 11 to 12% gels are shown. Relative sizes are shown in the center. Carefully cut membranes allow detection of pAkt (S473 or T308), pErk (T202/Y204), pS6 (S235/236), and 4E-BP1 (multiple sites). Loading controls, including total Akt or β -actin, can be detected following stripping of the blot.

protein: the most heavily phosphorylated species of 4E-BP1, labeled γ in Fig. 8.2, migrates the slowest, while the least phosphorylated species, labeled α , migrates fastest. A third, intermediately phosphorylated β form of 4E-BP1 can be detected migrating between the α and the γ forms. A stimulated sample in which 4E-BP1 is highly phosphorylated will show greater signal intensity in the top band when compared to an unstimulated or inhibitor-treated sample, which will show greater intensity in the middle and/or lowest band (Fig. 8.2).

Interpretation of immunoblotting with phospho-specific Abs requires confirmation that a greater or lesser signal intensity in one lane compared to another is because of differential phosphorylation levels and not simply because of a greater or lesser amount of total protein. Thus the inclusion of a loading control in any such experiment is crucial to the credibility of the results. In the case of 4E-BP1 or other proteins where phosphorylation is assessed by the ratio of bands with different migration distances, equivalent loading is less important.

4. PROTOCOLS FOR DETECTION OF PHOSPHOPROTEINS BY FLOW CYTOMETRY

Lymphocytes obtained from lymphoid organs of a mouse or peripheral blood of a human look homogeneous under a light microscope but are remarkably heterogeneous in gene expression and function. Within the general categories of T lymphocyte and B lymphocyte there are numerous subsets that have evolved to fulfill different roles in the adaptive immune system. Evidence is accumulating that differential signaling occurs in distinct subsets even when the same receptor is engaged (Benschop *et al.*, 1999, 2001; Li *et al.*, 2001; Tanaka *et al.*, 2003). Thus, immunoblot analysis of populations such as “total B cells” or “CD4⁺ T cells” examines a heterogeneous population, and cell types present in greater numbers will mask the response of the less numerous subsets. This is of special concern when comparing cells from individuals that have different ratios of these subsets. For example, many PI3K mutant mouse strains have altered lymphocyte development and exhibit altered percentages of T- or B-cell subsets compared to wild-type mice (Deane and Fruman, 2004; Donahue and Fruman, 2004; Koyasu, 2004; Okkenhaug and Vanhaesebroeck, 2003). Although it is possible to obtain relatively pure populations of individual subsets, this requires additional manipulations that can alter the response of the target cell type and often yields fewer cells than are needed for immunoblot analysis. These concerns call for a method that allows discrimination of these different subsets within unfractionated populations, and flow cytometry-based protocols have been established to that end.

Several laboratories (Chow *et al.*, 2001; Fleisher *et al.*, 1999; Krutzik *et al.*, 2005; Perez and Nolan, 2002) have developed methods to analyze the phosphorylation state of proteins of interest via flow cytometry (here abbreviated FACS for fluorescence-activated cell sorting). Several antibody supply companies (e.g., BD Biosciences, Cell Signaling) have developed and optimized antibodies for this approach, which has been termed “phosflow” or sometimes “phosphoflow.” In addition to allowing discrimination of different populations of cells within a single sample via staining with Abs directed against surface markers, the phosflow method requires far fewer cells than immunoblotting. Despite the lower cell number requirements, statistically robust data are obtained as a consequence of the collection of hundreds or thousands of individual cell events per subset analyzed. The approach also avoids potential postlysis artifacts that can occur in traditional methods, including but not limited to the action of cellular phosphatases and proteases. Heterogeneous cell populations can be stimulated en masse, and the responses of specific cell types within a sample can be quantitated and compared. This section presents two protocols for phosflow analysis of PI3K downstream readouts, describing the detection of pAkt, pErk, and pS6 by FACS.

4.1. Cell type discrimination by surface marker staining

A central consideration in phosflow analysis is choosing when to stain cells with antibodies to cell surface markers. Ideally, staining should take place after cell stimulation and fixation so that the binding of antibodies does not initiate signals that could confound interpretation of the results. However, some epitopes are masked or diminished by fixation. One approach is to stimulate cells followed by rapid cooling to 4° without fixation and then staining at this temperature before fixation. A caveat with this method is the potential for ongoing signaling (or phosphatase action) even at reduced temperatures. We have found that in many cases, staining chilled cells at the beginning of the experiment, before warming and addition of experimental stimuli, yields results that are equivalent to those obtained with cells stimulated before staining (data not shown). However, the surface markers used to distinguish the cell type(s) of interest should be chosen carefully, primarily to avoid those surface proteins known to initiate signaling when bound by specific Ab. An additional advantage of this approach is the ability to stain a batch of cells in bulk before splitting into different tubes for stimulation. This reduces time, antibody costs, and variability in staining. Staining of nonfixed cells before stimulation is a requirement when performing live cell-signaling measurements by FACS, for example, measurement of Ca²⁺ mobilization where signaling is measured in real time (Hess *et al.*, 2004).

As with most FACS experiments, markers should also be unambiguous whenever possible, providing clear delineation between populations. Be sure to test each chosen Ab, comparing staining of fixed cells to unfixed

cells, as we have found that some Ab-binding events do not survive the fixation process. In some cases this failure is fluorophore dependent, and the Ab will work if conjugated to a different fluorophore or if biotinylated and revealed with a streptavidin-conjugated secondary Ab. We incubate the cells with the FACS Abs for at least 15 min in Hanks' balanced salt solution on ice. Wash the cells at around 1100 rpm, resuspend them in media to 1×10^6 /ml, and aliquot into plates, microcentrifuge tubes, or even capped FACS tubes.

4.2. Inhibitor treatment, stimulation, and harvest of primary B lymphocytes

Pretreat cells for 15 min with PI3K inhibitors or vehicle and then stimulate the cells with a $2 \times$ volume of warmed mitogen as described earlier. Following stimulation, immediately transfer the cells to FACS tubes if necessary, centrifuge the cells at 1100 rpm for 5 min at 4° , and continue with the appropriate protocol.

4.3. Phosflow detection of pAkt and pErk

After harvest and centrifugation, resuspend the cells to 1×10^6 /ml in $1 \times$ PBS, add 16% MeOH-free formaldehyde (PolySciences) to a final concentration of about 2%, vortex briefly, and then incubate for 10 min in 37° water bath to fix the cells, then chill for 1 min on ice before spinning. When doing cell surface staining on fixed cells, this is the point after which cells are washed and stained with the appropriate antibodies. Spin the cells, decant the supernatant and resuspend in $100 \mu\text{l}$ incubation buffer (IB; $1 \times$ PBS, 0.5% BSA). Slowly add $90 \mu\text{l}$ ice-cold 100% MeOH while vortexing vortex briefly, and incubate on ice for 30 min to permeabilize the cells. Wash twice with 1 ml IB resuspend in IB, at about $50 \mu\text{l}/1 \times 10^6$ cells, and incubate for 15 min at room temperature. Then add primary phospho-specific Ab and incubate for 60 min at room temperature. We use the same phospho-specific Abs for Akt (S473) and Erk (T202/Y204) in phosflow as for immunoblotting. It is essential to determine the optimal concentrations of both primary and secondary Abs empirically for each cell type, beginning in the range of 1:50. Choose the concentrations that give the greatest increase in fluorescence intensity between stimulated and unstimulated or inhibitor-treated cells. We have found that for primary splenic B cells, 1:50 is the optimal dilution for both pAkt (S473) and pErk (T202/Y204) Abs. Wash the cells with 1 ml IB, resuspend in $100 \mu\text{l}/1 \times 10^6$ cells volume of secondary Ab, at the empirically determined dilution in IB, and incubate for 30 min at room temperature. For this protocol we make use of a goat anti-rabbit IgG secondary Ab conjugated to Alexa-488 (A488; Molecular Probes/Invitrogen); we have found the optimal dilution for primary B lymphocytes

to be 1:1000. Wash a final time with 1 ml IB and then resuspend to 1×10^6 /ml for acquisition. During preparation of the proofs of this article, we have improved the reliability of pAkt detection by including an amplification step. For a secondary antibody, we now use biotinylated donkey anti-rabbit (Jackson Immuno Research; 1:300 dilution). After 30 min, we wash again with 1 ml IB, then resuspend cells in streptavidin-APC (1:300 in 100 μ l of IB). After 20 min at 4°, continue with final wash and acquisition.

4.4. Phosflow detection of pS6

After harvest and centrifugation, resuspend the cells in 250 μ l CytoFix/CytoPerm fixation buffer (Becton-Dickinson kit) and incubate 20 min on ice. Wash once with 500 μ l staining buffer (SB; 1 \times PBS, 1% fetal bovine serum, 0.09% sodium azide, pH 7.4–7.6). When doing cell surface staining on fixed cells, this is the point after which cells are washed and stained with the appropriate antibodies. Wash twice with 500 μ l 1 \times perm/wash buffer (Becton-Dickinson kit) to permeabilize the cells. Resuspend after the final wash in 200 μ l 1 \times perm/wash buffer and add the same primary anti-pS6 Ab used for immunoblotting, at the empirically determined concentration. We use 0.3 μ l (1:600 dilution) of pS6 Ab per sample for splenic B cells. Incubate the cells for 20 min on ice, wash with 500 μ l 1 \times perm/wash, and resuspend in 200 μ l 1 \times perm/wash. Add the goat antirabbit IgG-FITC (Sigma) secondary Ab or the goat anti-rabbit IgG-A488 secondary Ab used earlier at the empirically determined concentration and incubate for 20 min on ice. For this protocol we prefer the FITC-conjugated Ab and use 0.3 μ l (1:600) per sample. Wash a final time with 500 μ l 1 \times perm/wash and resuspend in a 1×10^6 volume of 1 \times perm/wash for acquisition.

4.5. Data analysis and interpretation

An overlay of representative histograms for each Ab described is shown in Fig. 8.3. The PI3K dependence of phosphorylation of each protein is shown by the leftward shift of the peak when cells were pretreated with the inhibitor wortmannin. Where possible, additional inhibitors were used to demonstrate complete inhibition and give a background level of phospho-specific Ab binding, and in lieu of isotype controls, which often are not available for these sorts of experiments. As a control for pS6 we use the TORC1 inhibitor rapamycin (10 ng/ml), and for pErk we use the MEK inhibitor U0126 (10 μ M; from 10 mM stock concentration in ethanol). We find that in primary B cells, the peak shift of pAkt in stimulated cells is modest, but highly reproducible. The degree of separation between unstimulated and stimulated samples can be increased by optimizing the time period of cell “resting” at 37° before stimulation. A 1-h rest period works well. pErk and pS6 give more robust peak shifts, although these can also be

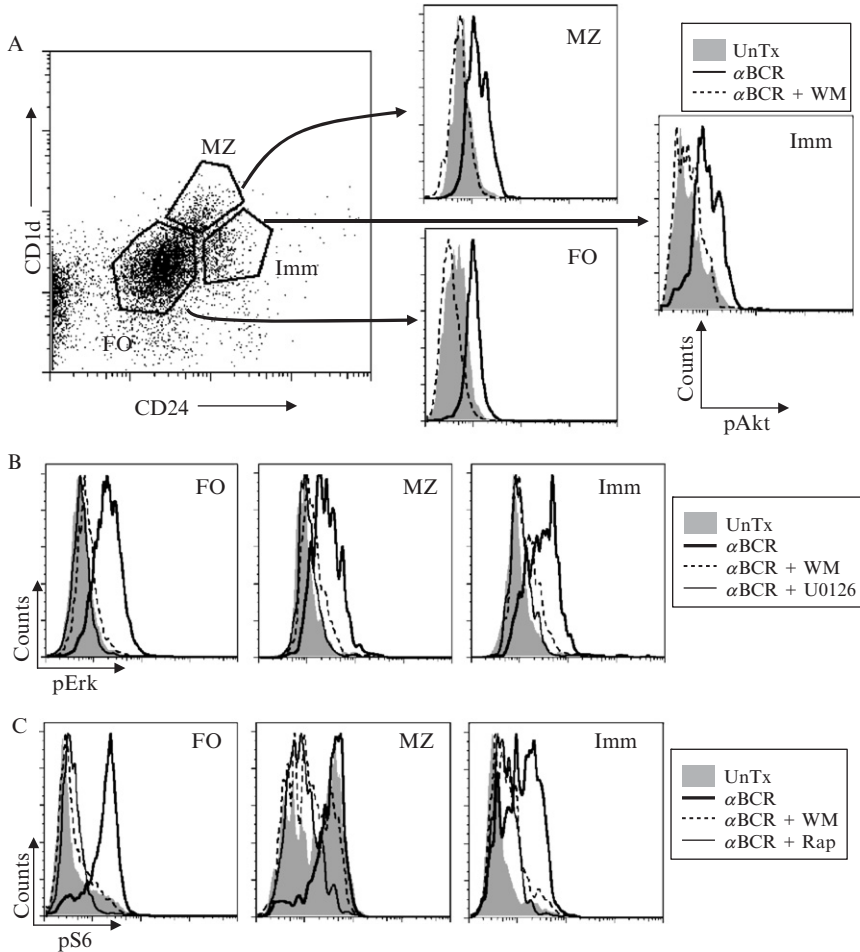


Figure 8.3 Phosflow detection of PI3K-dependent phosphorylation events. A representative experiment depicting analysis of splenic B-cell developmental subsets is shown. Total splenocytes are stained for surface markers CD24 and CD1d as shown in A, allowing differentiation among follicular cells (FO; CD24^{lo}/CD1d^{lo}), marginal zone cells (MZ; CD24^{int}/CD1d^{hi}), and the combined immature subsets (Imm; CD24^{hi}/CD1d^{int}) (Lyubchenko *et al.*, 2005). Cells are then stimulated via BCR cross linking, fixed, permeabilized, and incubated with the same phospho-specific Abs used for detection by immunoblot. (A) Histograms at right represent mean fluorescence intensity (MFI) of pAkt (S473) in FO, MZ, and immature cells. Note that stimulated cells display an increase in pAkt levels as shown by the rightward shift of the stimulated histogram (heavy line) when compared to unstimulated cells (filled histogram). This increase in pAkt levels is blocked by pretreatment with wortmannin (WM; dotted line), as evidenced by the leftward shift in the peak. The peak shift with pAkt (S473) is modest but highly reproducible. (B) Phosflow detection of pErk (T202/Y204) in BCR-stimulated B-cell subsets. The increase in MFI in stimulated cells (heavy line) with respect to unstimulated cells (filled histogram) indicates that Erk is phosphorylated in these cells,

influenced by the length of the rest period before stimulation (Donahue and Fruman, 2007). In splenic T cells, signal intensities are somewhat different, with pAkt detection more robust and pS6 less robust than in B cells. When analyzing data across several experiments, it must be kept in mind that settings vary from day to day in FACS experiments, which means that fluorescence intensity (MFI) values can also vary. It may be necessary, therefore, to calculate fold increases in MFI, with respect to unstimulated cells or some other control, in order to minimize the level of variation between experiments. When choosing gates to delineate separate subsets, it is critical to be conservative (i.e., edges of gates/regions should be well separated). Although restricting the gates reduces the number of cellular events analyzed, it increases the fidelity of subset discrimination. Robust data for even rare populations can be obtained by acquiring an appropriately large number of total cells.

5. DISCUSSION

Measuring PI3K-signaling activity in cells is a frequent goal of investigators in academic and pharmaceutical laboratories. There are a number of methods available to quantitate the PI3K lipid products themselves, and some of these are discussed in Guillou *et al.* (2007). For investigators studying PI3K signaling in primary lymphocytes, the available assays for measuring PtdIns(3,4,5)P₃ and other phosphoinositides directly are, as discussed later, insufficiently sensitive or selective to be readily applied in these cell types. Here we have provided protocols for measuring PI3K-dependent protein phosphorylation events as surrogate readouts of PI3K-signaling activity. We suggest that immunoblotting approaches are best applied to homogeneous cell populations such as lymphoma cell lines, whereas

whereas the PI3K dependence of this response is demonstrated by cells pretreated with wortmannin (dotted line). Pretreatment with the MEK inhibitor U0126 (thin line), which abolishes Erk phosphorylation in this system, establishes a baseline level of fluorescence and demonstrates specificity. (C) Phosflow detection of pS6 (S235/236) in BCR-stimulated B-cell subsets. FO and immature cells show low levels of pS6 in unstimulated cells with robust increases following BCR stimulation, whereas unstimulated MZ cells exhibit high levels of pS6, only slightly lower than those seen after activation. Further, wortmannin pretreatment blocks S6 phosphorylation almost completely in FO and immature cells, but less completely in MZ cells (Donahue and Fruman, 2007). It is the ability to detect these differences in rare subpopulations such as MZ cells that makes the phosflow method so powerful and important. Use of the mTOR inhibitor rapamycin (Rap) demonstrates specificity of the Ab by establishing a fluorescence background level that is TORC1 independent. Note that for each phosphorylation event measured, the cellular response is heterogeneous even within a given subset; that is, the histograms are not uniform. This is most apparent for cells in the marginal zone and immature gates, suggesting further phenotypic categories within these subsets.

FACS-based phosflow assays are particularly useful for analysis of primary lymphocytes where cell numbers are limiting and discrimination of signaling states among subsets is desired.

The phosflow approach carries additional advantages for studying PI3K signaling in lymphocytes, or indeed any signaling event that can be measured by this technique. Using flow cytometers capable of detecting multiple fluorophores, it is possible to analyze samples stained simultaneously for different phosphorylation events. Thus, relationships between different signaling events can be determined (Sachs *et al.*, 2005) and the aggregate signaling states of cellular subsets can be defined (Krutzik *et al.*, 2005; Perez and Nolan, 2002). Throughput can be enhanced and costs reduced by “bar coding” samples with different concentrations of fluorophores such as CFSE prior to pooling and bulk staining/analysis (Krutzik and Nolan, 2006).

One of the potential disadvantages of the phosflow approach is that it is difficult to prepare a “loading control,” that is, to normalize a phospho-specific signal to the total concentration of the target protein. The FACS approach circumvents loading issues in part because the same amounts of cell events are acquired even when total cell recovery differs among samples. Thus, when comparing the same cell type under two different treatment conditions, the total concentration of the target protein will generally be the same on a per cell basis. However, when comparing two different cell subsets, concentration of the target protein might differ. There are antibodies available to the nonphospho forms of the proteins described here (Akt, Erk, S6). However, most of these are polyclonal and the corresponding nonimmune sera are not as ideal as isotype controls for monoclonal antibodies. Nevertheless, the specific signal provided by phosphoAbs can be determined (even polyclonals) by using a cell sample treated with the appropriate pathway inhibitor. Hence, we favor the approach of calculating the change in MFI (Δ MFI) or the MFI fold change between samples \pm inhibitor and then comparing these values between experimental samples. In some cases it might be desirable to compare the percentages of cells above a particular fluorescence threshold rather than the MFI of the entire population. This approach applies more when only a subset of cells responds to the stimulus and the background of nonshifted cells dampens the detection of signal differences.

Another potential issue with phosflow is the ability of some phosphoAbs to recognize multiple proteins in a manner sensitive to the pathway inhibitor. For example, anti-pAkt might have some affinity for other phosphoproteins whose phosphorylation is wortmannin sensitive. For this reason, it is important to determine the overall specificity of a given antibody by probing an immunoblot containing whole cell extracts of the target cell type before and after stimulation \pm inhibitor (and visualizing a full range of molecular weights). The phosflow Abs described here are all highly specific when tested in lymphocytes by this approach (data not shown). There might be other antibodies that detect several bands whose phosphorylation is

wortmannin sensitive. However, if one is only looking for a general readout of PI3K pathway activity, it might be less important to study a single phosphoprotein than to study an aggregate “PI3K-dependent” signal.

As mentioned earlier, some investigators have used monoclonal antibodies directed to $\text{PtdIns}(3,4,5)\text{P}_3$ to quantitate this lipid in primary lymphocytes and cell lines by FACS (Anzelon *et al.*, 2003; Perez *et al.*, 2002, 2003; Sachs *et al.*, 2005). The obvious attraction of this approach is to measure the direct product of class I PI3K rather than a surrogate downstream readout. The commonly used antibody, available from Echelon Biosciences, appears to work quite well for fluorescence microscopy of adherent cells (Chen *et al.*, 2002; Niswender *et al.*, 2003). However, despite a significant effort in our laboratory and by several immunologist colleagues, we have been unable to validate this antibody for detection of $\text{PtdIns}(3,4,5)\text{P}_3$ generation in primary B or T cells. In some cases, the fluorescent signal decreases after cell activation, whereas in other cases there is an increased signal but it is not LY-sensitive. A representative experiment is shown in Fig. 8.4, which

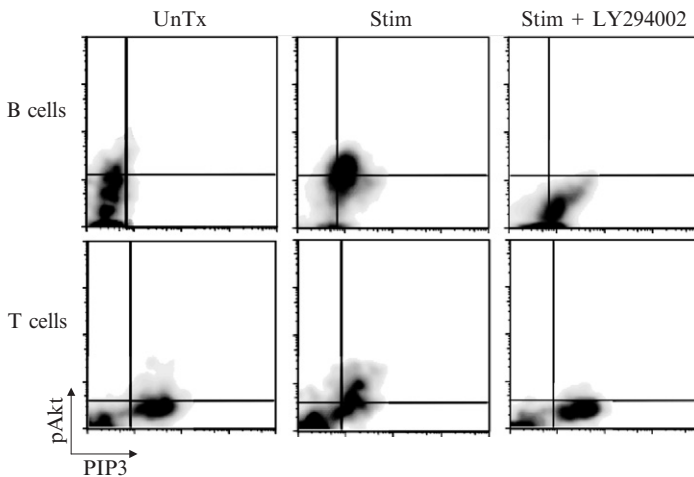


Figure 8.4 A monoclonal antibody to $\text{PtdIns}(3,4,5)\text{P}_3$ does not reveal specific increases in activated mature lymphocytes. Lymph node cells were incubated with unlabeled hamster mAb anti-CD3 to bind the T-cell antigen receptor complex. Cells were then aliquoted, warmed, and either left unstimulated (UnTx) or stimulated (stim) with either anti-IgM (B cells) or goat anti-hamster (T cells) to cross link the antigen receptors in the presence or absence of LY294002. After 1 min, cells were fixed, permeabilized, and stained with mouse mAb anti- $\text{PtdIns}(3,4,5)\text{P}_3$ (PIP3)-biotin and rabbit anti-pAkt, along with rat anti-B220-PE to differentiate between B and T cells. Cells were washed and then stained with secondary antibodies streptavidin-APC and anti-rabbit-Alexa488. Note that in B cells, the increase in $\text{PtdIns}(3,4,5)\text{P}_3$ is not LY-sensitive, whereas the increase in pAkt is blocked by this PI3K inhibitor. In T cells, the increase in pAkt is accompanied by a decrease in apparent detection of $\text{PtdIns}(3,4,5)\text{P}_3$. Similar results were obtained with different fixation/permeabilization protocols, concentrations of antibodies, and activation times.

depicts a two-color experiment in which the detection of pAkt in activated B and T cells increases in parallel to a LY-insensitive increase in PtdIns(3,4,5)P₃ (B cells) or a decrease in PtdIns(3,4,5)P₃ detection (T cells). To our knowledge, none of the published reports using this antibody in mature resting lymphocytes or Jurkat cells has shown data on the wortmannin/LY sensitivity of the observed fluorescence shifts (Anzelon *et al.*, 2003; Perez *et al.*, 2002, 2003; Sachs *et al.*, 2005). It may be that the antibody can detect PtdIns(3,4,5)P₃, as suggested by the enhanced signal in PTEN-deficient B cells (Anzelon *et al.*, 2003), but that it cross reacts with other cellular epitopes that mask the PI3K-dependent changes under physiological conditions of cell stimulation. The specificity of the antibody might also be cell type-dependent, as studies of neutrophils have suggested that robust, LY294002-dependent changes in fluorescence can be measured readily (Kuan *et al.*, 2006).

Other assay systems are available for measuring PtdIns(3,4,5)P₃ in cell extracts without radioactive labeling of the cells (Downes *et al.*, 2003). The specificity of these methods appears quite strong; however, it is not yet clear whether the approaches are sensitive enough to detect PtdIns(3,4,5)P₃ starting from reasonable cell numbers of primary lymphocytes. It is important to mention that elegant imaging techniques can be used to visualize the dynamics of PI3K lipid production and localization in primary lymphocytes and cell lines. The general approach is to use transfection or transgenesis to introduce green fluorescent protein-linked PH domain probes selective for PtdIns(3,4,5)P₃ or other phosphoinositides. Live cell fluorescence imaging can then be employed to examine the subcellular localization of the probes, with redistribution to the membrane an indication of lipid production (Costello *et al.*, 2002; Harriague and Bismuth, 2002). However, these approaches tend to be used more for qualitative assessments of lipid production as well as kinetic and spatial analyses. Until the development of PtdIns(3,4,5)P₃ quantitation assays of sufficient sensitivity and selectivity for lymphocytes, the downstream readouts described herein provide convenient correlates of PI3K-signaling output.

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