A Chemical Proteomics Approach to Phosphatidylinositol 3-Kinase Signaling in Macrophages*Ⓢ

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Prior work using lipid-based affinity matrices has been done to investigate distinct sets of lipid-binding proteins, and one series of experiments has proven successful in mammalian cells for the proteome-wide identification of lipid-binding proteins. However, most lipid-based proteomics screens require scaled up sample preparation, are often composed of multiple cell types, and are not adapted for simultaneous signal transduction studies. Herein we provide a chemical proteomics strategy that uses cleavable lipid "baits" with broad applicability to diverse biological samples. The novel baits were designed to avoid preparative steps to allow functional proteomics studies when the biological source is a limiting factor. Validation of the chemical baits was first confirmed by the selective isolation of several known endogenous phosphatidylinositol 3-kinase signaling proteins using primary bone marrow-derived macrophages. The use of this technique for cellular proteomics and MS/MS analysis was then demonstrated by the identification of known and potential novel lipid-binding proteins that was confirmed *in vitro* for several proteins by direct lipid-protein interactions. Further to the identification, the method is also compatible with subsequent signal transduction studies, notably for protein kinase profiling of the isolated lipid-bound protein complexes. Taken together, this integration of minimal scale proteomics, lipid chemistry, and activity-based readouts provides a significant advancement in the ability to identify and study the lipid proteome of single, relevant cell types.   *Molecular & Cellular Proteomics 6:1829–1841, 2007.*

In contrast to proteins and transcripts, intracellular phosphatidylinositol (PtdIns)[1] lipids are not genetically encoded. Of fundamental importance for understanding cell physiology and pathology, PtdIns lipids represent a rich resource of bioactive signaling molecules responsible for recruiting key protein effectors into signaling protein complexes (signalosomes). Although one of the major challenges is to study and map cell type-specific proteomes interacting with these lipid molecules, the study and identification of lipid-binding proteins has been limited to a few specialized groups. To date, most of the work has been devoted to protein-protein or DNA-protein interactions using well established "large scale protein fishing" methods including proteomics-based approaches. However, in contrast to DNA- and protein-mediated interactions, lipid-protein interactions and in particular those involving PtdIns are often weak making them difficult to study. An additional limitation with these studies is that experimental procedures cannot easily be scaled up. Until now, most studies have used affinity techniques and have led to the generation of lipid analogues used as chemical "baits." They have been performed using complex mixtures of cell types such as whole brain extracts from which total membrane fractions and cytosol were used. These studies have investigated the interaction of a distinct sets of proteins (1), the identification of inositol- (2, 3), receptor- (4), or a new phosphoinositide-binding protein (5) as well as proposed novel lipid binding selectivity for known interacting proteins (6). Few studies have been conducted toward large scale identification of PtdIns-interacting proteins (7) including only one mammalian study (8); however, this strategy has rarely been used in

[1] The abbreviations used are: PtdIns, phosphatidylinositol; LAC, lipid affinity chromatography; SEC, size exclusion chromatography; SEC-LAC, SEC followed by two-step LAC; SS-PtdIns, PtdIns containing inducible disulfide bond; SS-PtdIns(X)P$_n$, differentially phosphorylated SS-PtdIns (*e.g.* SS-PtdIns(3,4)P$_2$); B-SS-PtdIns(X)P$_n$, biotinylated SS-PtdIns(X)P$_n$; PI3K, phosphatidylinositol 3-kinase; BMDM, bone marrow-derived macrophage; IVK, *in vitro* kinase; IGK, in-gel kinase; NHS, N-hydroxysuccinimide; PNS, postnuclear supernatant; HEK, human embryonic kidney; PKB, protein kinase B; MSDB, mass spectrometry protein sequence database; HA, hemagglutinin; IGF1, insulin-like growth factor 1; PH, pleckstrin homology; Ilk, integrin-linked kinase; Btk, Bruton's tyrosine kinase; PDK1, phosphoinositide-dependent kinase 1; TCL, total cell lysate; CAP, cyclase-associated protein domain; Vav, VRG1-associated factor.

combination with protein kinase activity. Although these approaches have laid down invaluable groundwork, a major drawback is the requirement for large quantities of material in mammalian studies (8). Also more importantly, the cellular heterogeneity of the starting material has the disadvantage of complicating the precise assignment of newly identified proteins to a defined cell type. Therefore, with the difficult task of defining the lipid signalosome and the recent evidence for cell type-specific signal transduction of phosphatidylinositol 3-kinase (PI3K) isoforms (9), there is an increasing need to identify and study PtdIns-protein interactions in single cell types. The evidence that PI3K-dependent PtdIns signaling controls many processes including cytokine and radical oxygen production, phagocytosis, and directed cell migration (9–13) prompted us to use primary murine bone marrow-derived macrophages (BMDMs) as a model system. Because these cells yield only 1–2% of the protein used in a previous study (8), we further enhanced the methodology by engineering a novel lipid "bait" containing a cleavable disulfide bond (B-SS-PtdIns) that we show neither interferes with the lipid selectivity nor interferes with the protein function of the purified B-SS-PtdIns-bound protein complexes as evidenced by subsequent activity-based signal transduction experiments.

EXPERIMENTAL PROCEDURES

Additional information regarding the 3′-phosphorylated PtdIns(X)P$_n$ micelle preparation, *in vitro* lipid-protein and protein-lipid overlays, in-gel kinase (IGK) assay, and vectors to generate recombinant GST-fused proteins as well as details on nano-HPLC are described in the supplemental information.

Experimental Strategy—A scheme of the integrated chemical proteomics strategy is shown in Fig. 1*A*. Briefly the lipid affinity chromatography (two-step LAC) was first performed using novel soluble biotinylated PtdIns(X)P$_n$ ligands containing an inducible disulfide bond (B-SS-PtdIns(X)P$_n$). Specifically we used differentially phosphorylated B-SS-PtdIns(4,5)P$_2$, -(3,4)P$_2$, and -(3,4,5)P$_3$ to selectively isolate lipid-binding proteins (step 1) that were subsequently immobilized using streptavidin-coated beads. Captured proteins were eluted by a reductive cleavage of the disulfide bond (step 2) that connected the different PtdIns(X)P$_n$ head groups to the biotin moiety bound to the affinity matrix (Fig. 1*B*). This mild elution protocol preserved lipid-binding protein complexes for protein identification by MS/MS analysis as well as signal transduction experiments. Primary screen confirmation for potential novel PtdIns(X)P$_n$-binding proteins was performed *in vitro* using recombinant proteins.

Synthesis of Disulfide-containing Biotinylated Phosphoinositide Ligands—The procedures for synthesizing the SS-PtdIns(X)P$_n$ ligands (Fig. 1*C*, compounds **2**, **4**, and **6**) were analogous to those for preparing biotinylated PtdIns(X)P$_n$ ligands (Fig. 1*C*, compounds **1**, **3**, and **5**) as described previously (14). A solution of NHS-SS biotin (0.015 mmol, Sigma) in *N,N*-dimethylformamide (1 ml) was added to a solution of the amino PtdIns(X)P$_n$ (15) in 0.5 M triethylammonium bicarbonate (1 ml, pH 7.8), and the reaction was stirred overnight at room temperature. The solvents were evaporated *in vacuo*, and the residue was washed with acetone (4 × 2 ml). The crude product was dissolved in water (2 ml), applied to a column of DEAE-cellulose, and eluted with a gradient of 0–1 M triethylammonium bicarbonate. The desired fractions were pooled, lyophilized, and converted to the sodium salt with Dowex 50X8–100 (Na$^+$).

^{1}H (400 MHz) and ^{31}P (162 MHz) NMR spectra were recorded at 25 °C on a Varian INOVA instrument. Chemical shifts are given in ppm. Mass spectra were measured at the University of Utah Medicinal Chemistry Department using MALDI.

B-SS-PtdIns(3,4)P$_2$—^{1}H NMR (D$_2$O): 5.20 (m, 1H), 4.48 (dd, J = 7.6, 4.0 Hz, 1H), 4.28–4.36 (m, 2H), 4.25 (m, 1H), 4.08–4.18 (m, 2H), 3.80–4.00 (m, 4H), 3.70 (t, J = 9.2 Hz, 1H), 3.39 (t, J = 8.8 Hz, 1H), 3.17–3.22 (m, 5H), 3.07 (t, J = 6.4 Hz, 2H), 2.78–2.90 (m, 5H), 2.66 (d, J = 12.8 Hz, 1H), 2.53 (quart, J = 8.0 Hz, 4H), 2.28 (quart, J = 7.6 Hz, 4H), 2.13 (t, J = 7.6 Hz, 2H), 1.14–1.65 (m, 18H), 0.75 (t, J = 7.2 Hz, 3H). ^{31}P NMR (D$_2$O): 1.44, 0.94, 0.28. MALDI-MS, 1164.0 (M − H)$^-$.

B-SS-PtdIns(4,5)P$_2$—^{1}H NMR (D$_2$O): 5.17 (m, 1H), 4.50 (dd, J = 8.8, 4.8 Hz, 1H), 4.28–4.34 (m, 2H), 4.10–4.18 (m, 3H), 3.97 (t, J = 6 Hz, 2H), 3.85–3.91 (m, 2H), 3.80 (quart, 10 Hz, 1H), 3.66 (dd, J = 9.6, 2.4 Hz, 1H), 3.16–3.28 (m, 5H), 3.05 (t, J = 6.4 Hz, 2H), 2.80–2.91 (m, 5H), 2.65 (d, J = 12.8 Hz, 1H), 2.53 (quart, J = 6.8 Hz), 4H, 2.29 (quart, J = 7.6 Hz, 4H), 2.13 (t, J = 7.6 Hz, 2H), 1.10–1.62 (m, 18H), 0.74 (t, J = 7.2 Hz, 3H). ^{31}P NMR (D$_2$O): 1.99, 1.46, 0.33. MALDI-MS, 1164.0 (M − H)$^-$.

B-SS-PtdIns(3,4,5)P$_3$—^{1}H NMR (D$_2$O): 5.16 (m, 1H), 4.45 (dd, J = 7.6, 4.2 Hz, 1H), 4.23–4.38 (m, 4H), 4.12 (dd, J = 12.2, 8.6 Hz, 1H), 3.88–4.10 (m, 5H), 3.77 (t, J = 10.4 Hz, 1H), 3.13–3.20 (m, 5H), 3.03 (t, J = 6.4 Hz, 2H), 2.65–2.78 (m, 5H), 2.65 (d, J = 13.1 Hz, 1H), 2.51 (quart, J = 7.6 Hz, 4H), 2.26 (quart, J = 7.6 Hz, 4H), 2.10 (t, J = 7.6 Hz, 2H), 1.08–1.62 (m, 18H), 0.73 (t, J = 7.2 Hz, 3H). ^{31}P NMR (D$_2$O): 1.64, 1.19, 0.80, 0.18. MALDI-MS, 1244.1 (M − H)$^-$.

Two-step Lipid Affinity Chromatography—Primary differentiated macrophages isolated from mouse bone marrow were grown up to day 7 after animal sacrifice. BMDM cells were washed with ice-cold PBS, scraped, and centrifuged at 3,000 rpm. Cell pellets were resuspended in 1 ml of ice-cold hypotonic lysis buffer (10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl$_2$, 0.2 mM EDTA, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM Pefabloc, 40 mM β-glycerophosphate, 10 mM NaF, 1 mM sodium vanadate, 30 nM okadaic acid, 10 nM calyculin A, 1 mM benzamidine, and 25 nM microcystin) and incubated with rocking for 30 min at 4 °C. Lysates were further homogenized by eight passages through a 22-gauge needle syringe and centrifuged at 3,000 rpm to remove nuclei. NaCl (150 mM) and Nonidet P-40 (0.5%) were then added to the resulting postnuclear supernatant (PNS) and further incubated for 1 h at 4 °C under constant rotation. Preclearance was performed for 1 h with 50 μl of streptavidin beads prior to two-step LAC or with Protein A/G Plus-Sepharose beads prior to immunoprecipitations (Pierce). HEK cells were used only as proof of concept and treated as indicated under "Results."

Two-step LAC—For step 1, 1 ml of precleared protein lysates containing 1.2 mg of protein was mixed with 12 or 24 μM water-soluble C$_6$ biotinylated SS-PtdIns(X)P$_n$, respectively (Echelon Biosciences Inc. and Fig. 1*C*) for 5 h with rotation or with the indicated antibodies (immunoprecipitation). Lysates containing B-SS-PtdIns(X)P$_n$-bound proteins were then transferred onto 10-μl of packed streptavidin-coated beads and incubated 90 min with rotation followed by three washes with buffer containing 10 mM Hepes, 150 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors. For step 2, immobilized water-soluble C$_6$ biotinylated SS-PtdIns(X)P$_n$-bound protein complexes were eluted twice by addition of 15 μl of 30 μM DTT for 12 min at room temperature and separated by SDS-PAGE prior to MS/MS and/or immunoblotting. Immunoprecipitations were processed identically as described for two-step LAC but were stopped with regular sample buffer after the washing steps. For MS/MS protein identification following two-step LAC, primary differentiated macrophages were prepared as above, pooled together (6–10 mg), and separated by size exclusion chromatography (SEC). A small aliquot of the resulting enriched protein complex fractions was concentrated by protein precipitation and analyzed by immunoblotting to determine protein enrichment of known PtdIns(X)P$_n$-binding proteins. SEC fractions were pooled into four main pools according to this enrichment: Pool I, fractions 24–29;
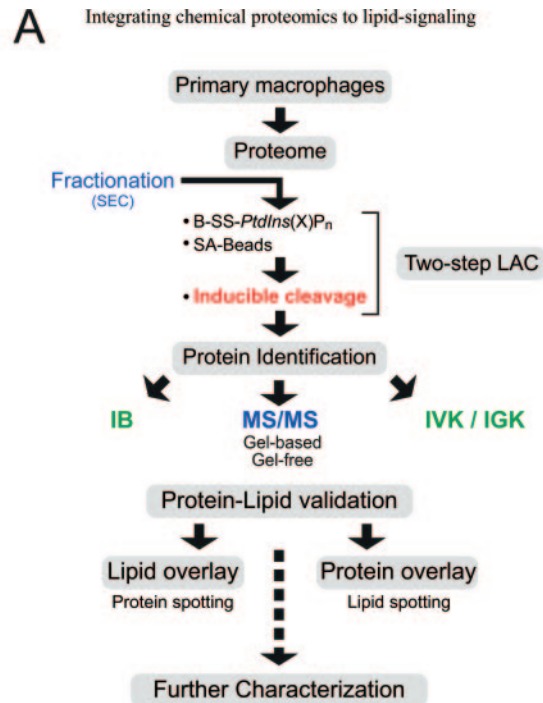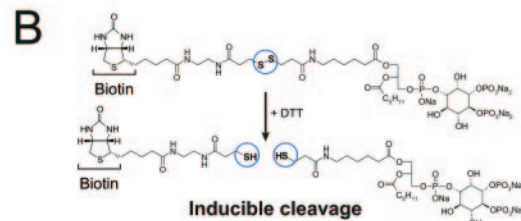
FIG. 1. *A*, diagram of the integrated experimental strategy: SEC, immunoblotting (*IB*), IGK assay, IVK assay, MS/MS, biotinylated disulfide phosphoinositide ligands (B-SS-PtdIns(X)P$_n$ ligands), streptavidin-coated beads (*SA-Beads*), and two-step LAC. *B*, example of the chemical structure of a biotinylated diC$_6$-biotin-SS-PtdIns(X)P$_n$ disulfide containing the 3′-phosphorylated PtdIns(3,4)P$_2$ and schematic of its cleavage induced by reduction with DTT. *C*, synthesis and structure of tethered phosphoinositides used as bait for the two-step LAC.
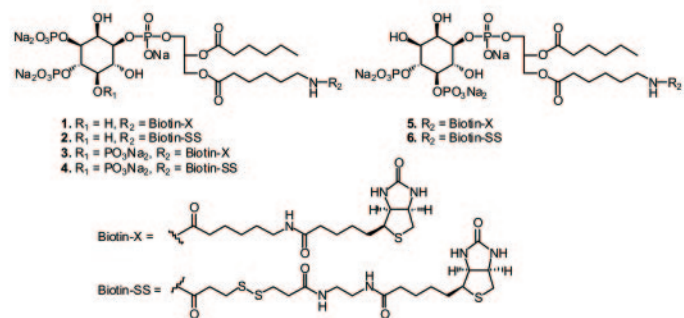
Pool II, fractions 30–41; Pool III, fractions 42–47; and Pool IV, fractions 48–53. The pools were supplemented with protease inhibitors and further submitted to the two-step LAC as described above. When crude lysate was compared with PNS as material source and processed for the two-step LAC, we observed no significant difference in the protein eluates except in the case of Akt/PKB, which showed lower protein recovery when PNS was used (data not shown).

In Vitro Kinase (IVK) Assay following Two-step LAC—Two-step LAC eluates were washed twice with buffer containing 10 mM Hepes, 150 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors followed by two washes with IVK buffer containing 50 mM Tris/HCl, pH 7.5, 50 mM NaCl, 7.5 mM of MnCl$_2$, 7.5 mM MgCl$_2$, 25 mM β-glycerophosphate, and 1 mM Na$_3$VO$_4$. The reaction was started by the addition of 30 μl of reaction mixture (10 μCi of [γ-^{32}P]ATP and 10 μM cold ATP in IVK buffer containing 2 mM DTT), run for 30 min at 30 °C, and stopped by addition of SDS sample buffer. Phosphorylation was monitored by phosphorimaging following SDS-PAGE using a Personal Molecular Image FX scanner (Bio-Rad).

Monitoring of Akt/PKB Activity following Two-step LAC—Two-step LAC protein complex eluates originating from insulin-like growth fac-

tor 1 (IGF1)-treated HEK-293 cells were assayed for Akt/PKB activity using the Akt kinase assay kit (Cell Signaling Technology, Beverly, MA) according to the manufacturer's instructions using cold ATP for the IVK reaction. Recombinant GSK3 (0.5 μg) was used as Akt/PKB kinase substrate, and phosphorylation was monitored by phospho-GSK3α/β (Ser-9/21) antibodies.

Protein Microsequencing: Nanoelectrospray-Tandem Mass Spectrometry of Silver-stained Proteins Resolved by SDS-PAGE—Two-step LAC eluates of separated proteins resulting from ammoniacal silver-stained bands obtained in the absence of cross-linker were excised and digested with trypsin as described previously (16) except that a modified porcine trypsin was used (Promega, Madison, WI) (17).

The digested mixture was resuspended in 10 μl of 5% formic acid and desalted on a capillary needle filled with 500 nl of POROS R2 chromatographic sorbent (Perseptive Biosystems, Framingham, MA). The washed peptides were eluted with 500 nl of 50% methanol in 5% formic acid into a disposable nanoelectrospray needle (Protana, Odense, Denmark). The needle was mounted onto the Z-Spray ion source of a Q-TOF mass spectrometer (Micromass, Manchester, UK), and the sample was extensively analyzed in the single MS and tandem MS modes. Doubly charged peptide ions observed in the MS spectra were analyzed in the tandem MS mode after selection through the quadrupole mass filter, and the masses of the product ions were analyzed by time-of-flight after fragmentation in the argon-filled collision cell.

The MS/MS spectra generated by the Q-TOF mass spectrometer were analyzed manually to obtain peptide sequence tags. The PeptideSearch Software (European Molecular Biology Laboratory; Mann and co-workers (18)) was used to search the MSDB using the manually generated tags to identify the peptide sequence and the protein.

Each spectrum was subsequently reanalyzed manually to compare the fragment ion peaks with a list of theoretical fragment ions obtained from the candidate sequence. This procedure allowed us to identify additional matching fragment ions in the spectra to further confirm the peptide sequence.

Identification from Non-stained Protein Gels: Nano-HPLC-MS—Briefly two-step LAC eluates containing lipid-binding protein complexes were separated by SDS-PAGE for a short period of time (4–5 cm). The area in which the protein had migrated was entirely cut out, sliced, reduced with DTT, carboxymethylated using iodoacetamide, and digested with trypsin. Tryptic peptides were extracted with formic acid and separated by nano-HPLC (LC Packings, Amsterdam, The Netherlands) on a PepMap C_{18} column. The eluate of the column was applied on line to an LCQ Deca XP ion trap mass spectrometer (Thermo Finnigan) equipped with a home-made nanospray source. Additional details are described in the supplemental information. The peak lists were extracted from the DecaXP raw files using Bioworks 3.1. The peak list (dta) files were then merged together with a Mascot Perl script (merger.pl revision 14) into a file (mgf) (Matrix Science).

The search was performed using Mascot revision 1.9.05. The enzyme specificity was trypsin with one missed cleavage allowed. The peptide modifications that were allowed were a fixed carbamidomethylation of cysteines and a variable modification of oxidized methionine. The mass tolerance for the precursor ions was 3 and 0.8 Da for the fragment ions.

The search was conducted on two databases, MSDB (November 2003) and mouse Ensembl Database Build 33 (2002) (www.ensembl.org). We used the mouse genome to enable rapid mapping of each identified peptide and as a means to identify protein families. An analysis on MSDB (no species restriction) was performed to remove contaminants such as keratins and trypsin peptides. The total number of sequences that was searched in the mouse Ensembl Database was

32,284, and the total number of sequences that was searched in the MSDB was 1,268,262. We selected peptides that reached a score of 40 and above; however, if a peptide score was just below that threshold a manual inspection was performed.

The use of the mouse Ensembl genome allowed for the identification of peptides that could highlight several members of a protein family. In the case of a multiple family detection, we did not include this within our validation sets to avoid cloning of the entire protein family.

Reagents—Recombinant pure amino-terminally tagged His-Akt/PKB and His-Akt/PKBΔPHD proteins were purchased from Upstate Biotechnology (Lake Placid, NY). Recombinant GSK3 fusion protein was purchased from Cell Signaling Technology.

Recombinant Proteins—For expression and purification of GST-proteins (PRDX1, CAP1, and Rab5c), *Escherichia coli* cultures were grown in LB medium with 50 μg/ml ampicillin at 37 °C for 4 h. Protein expression was induced by addition of 0.1 mM isopropyl 1-thio-β-D-galactopyranoside, and cultures were further grown at 28 °C for 4 h. Bacterial pellets were resuspended in 25 ml of PBS, pH 7.5, and lysed by sonication. After centrifugation at 9,000 $\times$ *g* for 30 min, GST-proteins were purified from the supernatants with glutathione-Sepharose 4 Fast Flow resin (Amersham Biosciences) using the manufacturer's instructions.

Transfection and Plasmids—Epitope-tagged Akt/PKB was transfected on HEK-293 cells with FuGENE 6 transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. HA-Akt/PKB subcloned into pCDNA3 for mammalian expression has been described elsewhere (19).

Cell Growth and Stimulation—Primary BMDM preparation and growth have been described elsewhere (20). Signaling experiments were performed at day 7 with differentiated adherent BMDM cells (3×10^7 in 15-cm plate) previously characterized by fluorescence-activated cell sorting (BD Biosciences) for CD11b, CD11c, GR-1, F4/80, and CCR5 expression (data not shown). After starvation for 3 h in serum-free medium (without L-929 differentiating medium) cells were stimulated with IGF1 (3 min; 100 ng/ml) (Sigma). HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (nutrient mixture F-12) containing 10% fetal bovine serum and antibiotics.

Antibodies and Immunoblots—Following protein transfer or antibody-based overlays, Hybond-C Extra nitrocellulose membranes (Amersham Biosciences) were incubated with one of the following antibody solutions: rabbit anti-Akt/PKB, -phospho-Akt/PKB Ser-473, -phospho-Akt/PKB Thr-308, or -phospho-GSK3 Ser-9/21 (Cell Signaling Technology); mouse anti-HA.11 (Babco, Richmond, CA); mouse anti-Bruton's tyrosine kinase (Btk), anti-integrin-linked kinase (Ilk), and anti-phosphoinositide-dependent kinase 1 (PDK1) and rabbit anti-VRG1-associated factor (Vav) (BD Transduction Laboratories); rabbit anti-Tec (Upstate Biotechnology); and mouse anti-GST (Molecular Probes, Leiden, The Netherlands). UltraLink immobilized Protein A/G Plus and streptavidin beads were from Pierce. Proteins were visualized by ECL (Amersham Biosciences) following incubation with the appropriate antibodies.

RESULTS

Selective Capture of Cellular PtdIns-binding Proteins by Two-step LAC—The pivotal role of the pleckstrin homology (PH)-containing protein kinase Akt/PKB in PI3K-mediated signaling and its low protein expression (19, 21) motivated our choice to use this protein as a reference for the validation of our experimental strategy. We first tested HEK-293 cells over-expressing HA epitope-tagged Akt/PKB (HA-Akt/PKB) as a raw source of material in combination with either of the three
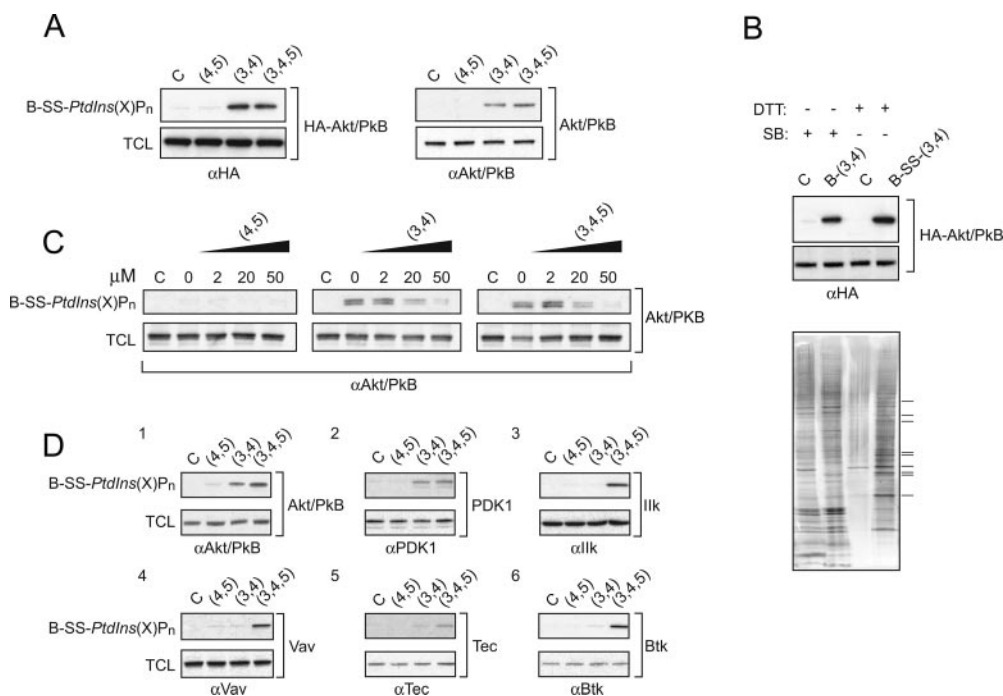
Fig. 2. **PtdIns(X)P$_n$-selective isolation of cellular signaling proteins by two-step LAC using biotinylated B-SS-PtdIns(X)P$_n$.** *A*, isolation of HA epitope-tagged Akt/PKB from HEK-293 cells (*top left*) or endogenously expressed in primary BMDMs (*top right*) following DTT elution. Fifteen micrograms of TCLs prior to two-step LAC are shown as a control for loading. *C*, control streptavidin beads without bait; *(4,5)*, B-SS-PtdIns(4,5)P$_2$; *(3,4)*, B-SS-PtdIns(3,4)P$_2$; *(3,4,5)*, B-SS-PtdIns(3,4,5)P$_3$. *B*, recovery of recombinant HA-Akt/PKB expressed in HEK-293 cells after affinity binding to non-cleavable B-PtdIns(3,4)P$_2$ or cleavable B-SS-PtdIns(3,4)P$_2$ followed by elution with sample buffer (*SB*) or DTT as indicated. The *middle panel* corresponds to starting material prior to two-step LAC following immunoblotting with anti-HA antibodies and is shown as a control. The *upper panel* corresponds to HA epitope-tagged Akt/PKB following immunoblot analysis with anti-HA antibodies. The *lower panel* corresponds to the silver staining of the two-step LAC eluates containing proteins after SDS-PAGE separation. *Horizontal lines* point to protein bands either enriched or only present in the DTT-eluted samples. *C*, inhibition of the binding of endogenous Akt/PKB to 2 μM B-SS-PtdIns(X)P$_n$ by increasing concentrations (2–50 μM) of their corresponding non-biotinylated analogues, PtdIns(X)P$_n$ ligands lacking the disulfide bond, performed in HEK-293 cells. *D*, selective endogenous isolation of (6) cellular PtdIns(X)P$_n$-binding proteins. Primary BMDM cell extracts were submitted to two-step LAC as described under "Experimental Procedures" followed by immunoblot analysis with specific antibodies against Akt/PKB, PDK1, Ilk, the exchange factor Vav, Tec kinase (*Tec*), and Btk. Results are representative of a minimum of three independent experiments.

B-SS-PtdIns(X)P$_n$ ligands, -PtdIns(4,5)P$_2$, -PtdIns(3,4)P$_2$, or -PtdIns(3,4,5)P$_3$, used as "lipid baits." The use of tagged protein allowed optimization of the experimental conditions by monitoring two-step LAC efficiency and B-SS-PtdIns(X)P$_n$ functionality. This monitoring was performed by immunoblotting HA-Akt/PKB on the resulting DTT eluates (Fig. 2*A*, *left panel*). Because the original goal was to isolate endogenously expressed PtdIns(X)P$_n$-binding proteins in primary cells, we repeated the experiment using macrophages and successfully isolated endogenous Akt/PKB with equal PtdIns(X)P$_n$ binding selectivity (Fig. 2*A*, *right panel*). We then compared the efficiency of B-SS-PtdIns(3,4)P$_2$ with its analogue lacking the cleavable disulfide bond, B-PtdIns(3,4)P$_2$, for isolation of Akt/PKB. As shown in Fig. 2*B* (*top panel*) the lower intensity of the Akt/PKB signal corresponding to the elution by sample buffer from B-PtdIns(3,4)P$_2$, for a similar signal in the total cell lysates (*middle panel*), demonstrates that the disulfide bond-containing ligand not only showed the same selectivity but also higher sensitivity for Akt/PKB when the cleavable system

was used to detach proteins from the bait compared with the corresponding non-SS bond-containing analogue (compare *lanes 2* with *lanes 4*). To further establish the utility of the two-step LAC method for protein identification by mass spectrometry and evaluate the overall protein content of the purified eluates, we next performed SDS-PAGE separation of the same samples and stained the resulting gel with silver. Elution with DTT recovered a substantially lower proportion of non-specific proteins as indicated by the significantly lower protein content in the DTT elution compared with the sample buffer elution of the control beads with no ligand (Fig. 2*B*, silver gel, compare *lane 1* with *lane 3*). Consequently after sample buffer elution, control beads and beads with B-PtdIns(3,4)P$_2$ displayed virtually the same protein pattern on the gel (compare *lane 1* with *lane 2*), whereas in the elution with DTT, the control sample showed considerably less protein than the DTT elution of the B-SS-PtdIns(3,4)P$_2$ sample (compare *lane 3* with *lane 4*). The absence of difference between *lanes 1* and *2* demonstrates that sample buffer elution was largely non-selective
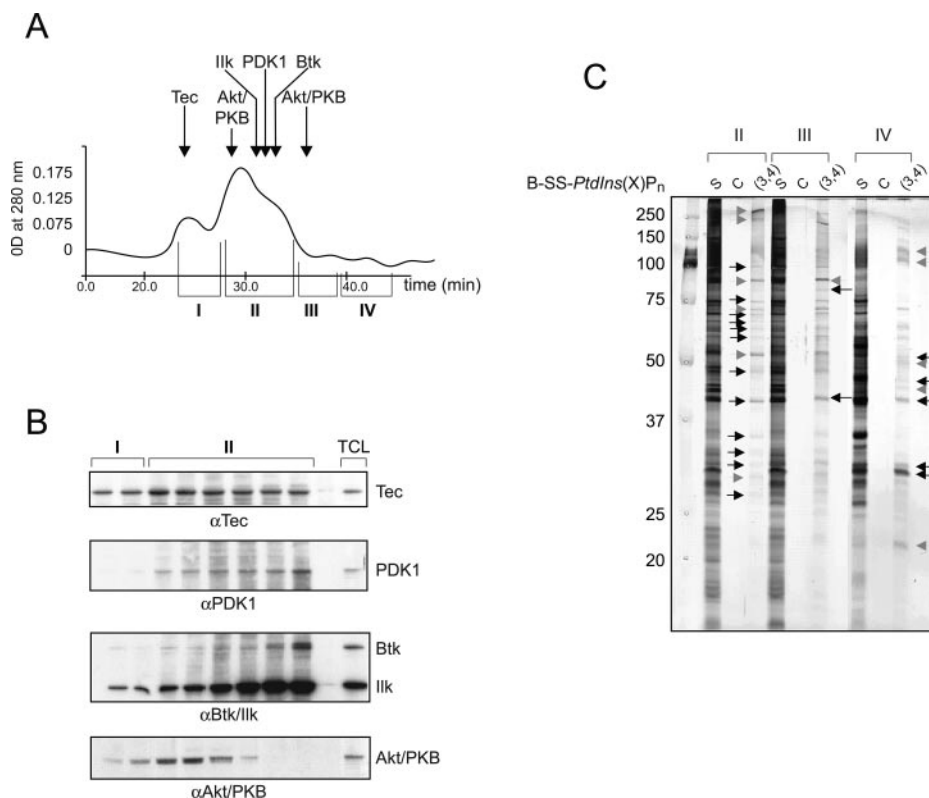
FIG. 3. **Identification of PtdIns-binding proteins from primary macrophages by SEC-LAC.** *A*, SEC of a BMDM cell extract through a Superdex 200 gel filtration column. The figure shows the 280 nm protein elution profile obtained during a 1-h run. *I–IV* indicate the pooled fractions chosen for further two-step LAC described under "Experimental Procedures." *Arrows* indicate the different retention times for individual B-SS-PtdIns(*X*)P$_n$-binding proteins. *B*, immunoblot analysis of the indicated SEC pooled fractions after SDS-PAGE. The immunoblot shows fractions corresponding to pools I and II (fractions 24–41). TCL prior to SEC was used as an index for protein enrichment. *C*, analytical SDS-PAGE stained with ammoniacal silver showing protein recovery after SEC-LAC using B-SS-PtdIns(3,4)P$_2$ and DTT elution. *S*, sample corresponding to the SEC pool and representing the original material for the two-step LAC; *C*, control showing the DTT elution of a two-step LAC performed with streptavidin beads lacking B-SS-PtdIns(3,4)P$_2$; *(3,4)*, B-SS-PtdIns(3,4)P$_2$. *Arrows* point to protein bands from this particular run and further submitted to microsequencing. *Grey arrows* indicate proteins enriched following SEC-LAC. Results are representative of a minimum of three independent experiments.

and thus could not provide samples suitable for selective protein identification by MS/MS. In contrast, B-SS-PtdIns-(3,4)P$_2$ eluates not only allowed a complete isolation of protein candidates with less nonspecific protein recovery but also displayed several intense protein bands that were absent when the B-PtdIns(3,4)P$_2$ matrix lacking the SS bond was used (Fig. 2*B*, *lower panel*, *horizontal lines*). We further assessed the potency and selectivity of the disulfide lipid baits (Fig. 2*C*) to capture target proteins by testing their binding to endogenous Akt/PKB in the presence of increasing concentrations of their corresponding non-biotinylated analogues added at the same time as B-SS-PtdIns(*X*)P$_n$. In accordance with earlier literature, Akt/PKB selectively associated with B-SS-PtdIns(3,4)P$_2$ and B-SS-PtdIns(3,4,5)P$_3$ but not with B-SS-PtdIns(4,5)P$_2$ (22) (used here as internal PtdIns bait control) in a concentration-dependent manner.

To benchmark the two-step LAC approach for more than Akt/PKB and further validate the specificity and efficacy of the B-SS-PtdIns(*X*)P$_n$ lipids, we searched for other known PI3K-regulated PtdIns-interacting proteins expressed endogenously in primary macrophages. We first identified and confirmed the B-SS-PtdIns(*X*)P$_n$ binding of PDK1, displaying lipid selectivity identical to that of Akt/PKB (included as a control for comparison) with PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ (23) (Fig. 2*D*, *panels 1* and *2*). We also confirmed Ilk as a PtdIns(3,4,5)P$_3$-binding protein previously described in cell-based assays (24) (Fig. 2*D*, *panel 3*) and the Vav-1 exchange factor, which is exclusively expressed in hematopoietic cells and preferentially binds to PtdIns(3,4,5)P$_3$ (25) (Fig. 2*D*, *panel 4*). Similarly Tec kinase (Fig. 2*D*, *panel 5*) as well as the Tec family kinase Btk (26) (Fig. 2*D*, *panel 6*) displayed selective binding to PtdIns(3,4,5)P$_3$. In summary, several independent experiments showed that Akt/PKB and PDK1 specifically recognized PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ with similar affinity, whereas Tec, Btk, Ilk, and Vav preferentially associated with PtdIns(3,4,5)P$_3$.

Interestingly we failed to selectively detect Wasp, paxillin, and Sos as B-SS-*PtdIns*(*X*)P$_n$-binding proteins despite their

presence in variable quantities in the proteome fractions obtained by SEC (data not shown). Thus, this demonstrates that the experimental conditions we used are not suitable for all PtdIns(X)P$_n$-binding proteins. This could be due in part to the use of the short sn-2-C$_6$ acyl chain and the sn-1-acyl chain (Fig. 1C) with multiple connecting amides and a biotin group, which introduce variables that affect some PtdIns(X)P$_n$-protein interactions more than others.[2] These concerns notwithstanding, the two-step LAC using soluble, cleavable B-SS-PtdIns(X)P$_n$ ligands allowed the efficient isolation of low abundance, endogenously expressed PtdIns(X)P$_n$-binding proteins in a single relevant cell type and with high ligand selectivity for a given lipid bait. Additional evidence of B-SS-PtdIns(X)P$_n$ selectivity is illustrated by SDS gel electrophoresis and silver staining (see Supplemental Fig. 1), a prerequisite for the identification of lipid-binding proteins by MS/MS analysis.

Microsequencing Protein Identification from Sequential SEC and Two-step LAC—Protein identification was performed using B-SS-PtdIns(3,4)P$_2$ as the principal bait, although B-SS-PtdIns(3,4,5)P$_3$ and B-SS-PtdIns(4,5)P$_2$ were also used as internal controls for lipid selectivity as indicated under "Results" and in the supplemental information (Supplemental Fig. 1). In addition to its key role in the PI3K signaling pathway, the observation that some stimuli induce large increases in PtdIns(3,4)P$_2$ levels without affecting the levels of PtdIns(3,4,5)P$_3$ (27) suggests that PtdIns(3,4)P$_2$ may induce signaling pathways distinct from those induced by PtdIns(3,4,5)P$_3$ (28). Furthermore macrophages impaired in PtdIns(3,4)P$_2$ production show several cellular defects, a reduced number of bone marrow lymphoid and erythroid progenitor cells as well as lung infiltrates and a shorter life span (29, 30).

In an attempt to sieve for primary macrophage protein complexes of medium to small sizes, we performed an analytical SEC prior to the two-step LAC described above. A pore size of 200 kDa allowed for the separation and enrichment of several preidentified B-SS-PtdIns(X)P$_n$-binding proteins (Fig. 3A). The enrichment was either moderate (PDK1 and Akt/PKB) or substantial (Btk, Tec, and Ilk) as evaluated by comparing the corresponding immunoblot signals from the total cell lysates (TCLs) before SEC with those from SEC-positive fractions (Fig. 3B). Based on these results, positive fractions were collected into four main pools (I–IV) that were then applied to the two-step LAC to identify novel PtdIns(X)P$_n$-binding proteins. Fig. 3C shows a representative silver staining of an SDS-PAGE gel corresponding to an example of the two-step LAC eluates obtained after SEC (SEC-LAC) from primary macrophages originating from only 6 mg of total protein. Some protein candidates were significantly enriched (*II–IV*, *grey arrows*) relative to the starting material (*S*). As seen in Fig. 3C, the relative lack of sample complexity and the

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[2] C. Pasquali, R. Booth, C. G. Ferguson, G. D. Prestwich, M. Camps, and C. Rommel, unpublished observations.

TABLE I
Identification of PtdIns(X)P$_n$-binding proteins: known lipid-binding proteins

A list of known PtdIns-binding proteins isolated by two-step LAC (IB) or SEC-LAC (MS/MS) as indicated by the identification method (ID) is shown.

Name	UniProt ID	ID	Ptdins(X)P$_n$ interaction
Akt/PKB		IB	PH domain
Ilk		IB	PH domain
Btk		IB	PH domain
PDK1		IB	PH domain
Tec		IB	PH domain
Vav		IB	PH domain
Gelsolin	GELS_MOUSE	MS/MS	Charged/domain[a]
Talin	TLN1_MOUSE	MS/MS	Charged/domain[a]
GAP1 (IP4BP)	RASA3_MOUSE	MS/MS	PH domain[b]
Hexokinase II	HXK2_MOUSE	MS/MS	Not determined[b]

[a] Proteins identified using B-SS-PtdIns(4,5)P$_2$ and B-SS-PtdIns(3,4)P$_2$.
[b] Only with B-SS-PtdIns(3,4)P$_2$. PtdIns(X)P$_n$-interaction indicates the described mechanism of interaction with lipids.

quality of band resolution with respect to the limited starting material were already sufficient for gel-based protein identification by MS/MS.

Our MS/MS analysis identified gelsolin (GELS_MOUSE), GAP1/IPB4P (RASA3_MOUSE), hexokinase2 (HXK2_MOUSE), and talin (TLN1_MOUSE) (Tables I and II), which have been described previously as selective PtdIns(X)P$_n$-binding proteins (7, 31–33) further validating our strategy for protein identification by MS/MS analysis. Altogether the combination of the cleavable B-SS-*PtdIns*(X)P$_n$ lipid reagents with the analytical SEC enabled us to avoid preparative scale-up. This combination is further supported by the improvement of the signal to noise ratio (compare Figs. 2B and 3C) and the selective enrichment of several protein bands (Fig. 3C, compare "S" with "$(3,4)$"; *grey arrows*) used primarily for protein identification by MS/MS analysis.

To broaden the identification of the content of B-SS-PtdIns(X)P$_n$ affinity-purified protein complexes present in the eluates and increase the protein recovery, we then performed a further protein separation. This time, however, the protein front was allowed to migrate longer for 1 h before processing for protein identification (omitting the protein staining step). This allowed us to identify and select a novel set of PtdIns(X)P$_n$-binding candidates. Importantly as the quantity of material is a limiting factor in most proteomics approaches that use biologically relevant cell sources, we never exceeded the analytical scale (10 mg of starting material) making the two-step LAC applicable to virtually any primary cell type. A list of these proteins is shown in Table III. For single peptide-based protein identifications, additional details are shown in Supplemental Table D.

Protein Classes Identified by Mass Spectrometry—To distinguish the potential novel direct B-SS-PtdIns-binding pro-

TABLE II

Identification of PtdIns(X)P_n-binding proteins

Additional information on known PtdIns(X)P_n-binding protein identified by MS/MS is shown. MGI, Mouse Genome Informatics database code and identification. Other references to the identified proteins are added (Ensembl genes as well as UniProt identifier). Coverage (%), sequence coverage was calculated using the mature protein sequence. Score(s), score for each identified peptide. NA, not applicable (peptides identified using peptide sequence tag). Peptides (n), number of unique peptides identified.

MGI code	MGI ID	Ensembl gene ID	Coverage %	Score(s)	Peptides (n)	UniProt ID	Description	Domains
MGI:95851	Gsn	ENSMUSG00000026879	1.7	NA	1	GELS_MOUSE	Actin-depolymerizing factor, gelsolin	Gelsolin, actin domain
MGI:1099832	Tln1	ENSMUSG00000028465	0.6	NA	1	TLN1_MOUSE	Talin	Actin binding domain (I/L WEQ)
MGI:1015197	Hk2	ENSMUSG00000000628	3.1	57/47	2	HXK2_MOUSE	Hexokinase II	HK domain
MGI:1197013	Rasa3	ENSMUSG00000031453	1.3	58	1	RASA3_MOUSE	Ras GtPase-activating 3 GAP1 (IP4BP INS P4 binding)	GTP binding, Ras transforming

TABLE III

Identification of PtdIns(X)P_n-binding proteins: potential new lipid-binding proteins

A set of selected potential new PtdIns(X)P_n-binding proteins is shown. MGI, Mouse Genome Informatics database code and identification. Other references to the identified proteins are added (Ensembl genes as well as UniProt identifier). Coverage (%), sequence coverage was calculated using the mature protein sequence. Score(s), score for each identified peptide. NA, not applicable (peptides identified using peptide sequence tag). Peptides (n), number of unique peptides identified.

MGI code	MGI ID	Ensembl gene ID	Coverage %	Score(s)	Peptides (n)	UniProt ID	Description	Domains
MGI:1345961	Coro1a	ENSMUSG00000030707	7.8	62/47/43	3	COR1A_MOUSE	Coronin-like protein p57	5 WD repeat, coiled/coiled
MGI:95772	Gnai2	ENSMUSG00000035562	6.8	49/44	2	GNAI2_MOUSE	Guanine nucleotide-binding protein G_i, α 2 subunit	Prenyl site binding CAAX box
MGI:2385902	Picalm	ENSMUSG00000039361	4.8	56/76	2	PICA_MOUSE	Phosphatidylinositol-binding clathrin assembly protein	Epsin N-terminal domain
MGI:104808	Lcp1	ENSMUSG00000021998	5.3	56/42	2	PLSL_MOUSE	65-kDa macrophage protein, L-plastin	Actin-binding actinin domain, calponin-like
MGI:105306	Rab5c	ENSMUSG00000019173	5.6	86	1	RAB5C_MOUSE	Ras-related protein Rab-5C	GTP-binding, Ras transforming
MGI:2145823	Kctd12	ENSMUSG00000041633	36.5	54/44/55/46	4	Q8CBQ4_MOUSE	Riken clone: 9530062N09	K tetramerisation domain BTB/POZ
MGI:99523	Prdx1	ENSMUSG00000025733	15.6	40/47/49	3	PRDX1_MOUSE	Macrophage 23-kDa stress protein, peroxidredoxin-1	Thioredoxin-like
MGI:88262	Cap1	ENSMUSG00000028656	11.8	69/67/52/40/55	5	CAP1_MOUSE	Adenylyl cyclase-associated protein 1	CAP domain
MGI:2150641	Edg8	ENSMUSG00000045087	2.3	NA	1	EDG8_MOUSE	Sphingosine 1-phosphate receptor Edg-8	G protein receptor
MGI:1928344	Vps29	ENSMUSG00000029462	7.1	43	1	VPS29_MOUSE	Vacuolar protein sorting-associated protein 29	Transmembrane
MGI:95784	Gnb2	ENSMUSG00000029713	9.7	49/41/44	3	GBB2_MOUSE	Guanine nucleotide-binding protein $G_{(i)}/G_{(s)}/G_{(t)}$ subunit $\beta2$	WD repeat

teins described in Table III from indirect interactions originating from multiprotein complexes, we performed literature searches for proteins carrying lipid-binding modules and further investigated their molecular characteristics. Interestingly most of the potential novel PtdIns(X)P$_n$ targets contained other known lipid-interactive modules, such as C*AAX* box, p67 Phox homology, CAP, or coiled coil domains, but have not been connected previously to PI3K-regulated PtdIns(X)P$_n$ signaling. Examples are CAP1 (CAP1_MOUSE), a protein involved in G-protein-mediated cell polarization and actin dynamics during leukocyte chemotaxis (34); the small GTPase Rab5c (RAB5C_MOUSE), previously shown to interact directly with PI3K (35, 36); and the macrophage 23 stress protein (PRDX1_MOUSE), a member of the peroxiredoxin family, which protect cells against oxidant-induced plasma membrane damage (37).
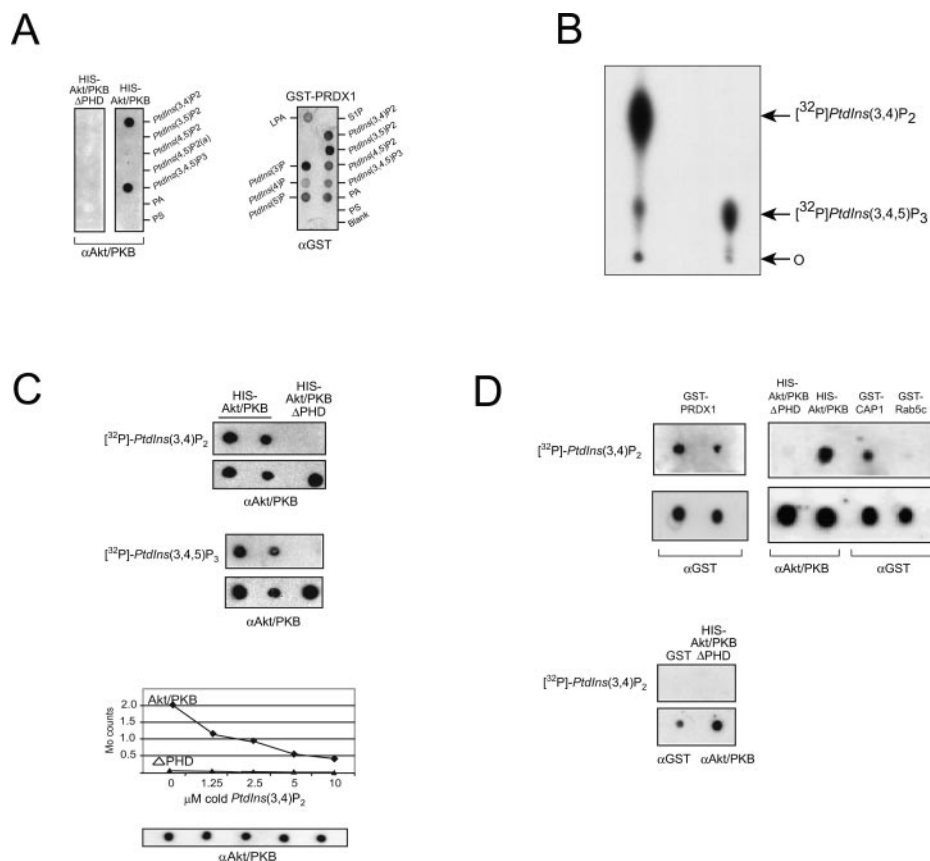
Validation of Direct Lipid-Protein Interaction—To validate experimentally the potential novel lipid-binding protein interaction with one or more lipids, we cloned and tested the three candidate proteins described above. For this, we used a protein overlay assay that uses conventional PIP Strips™ where nitrocellulose membranes preadsorbed with various lipids including PtdIns are incubated with the recombinant tagged proteins of interest. As expected, full-length recombinant His-Akt/PKB used as a positive control selectively associated with its two 3′-phosphorylated PtdIns(X)P$_n$ lipids, whereas the negative control His-Akt/PKBΔPHD (a mutant of Akt/PKB lacking the PH domain lipid-binding module) did not interact with any of them (Fig. 4*A*, *left panels*). We then tested our three candidate proteins (GST-PRDX1, GST-CAP1, and GST-Rab5c) and observed that only PRDX1 reproducibly showed a direct and heterogenous lipid association (Fig. 4*A*, *right panel*). As we experienced several reproducibility issues using PIP Strips with the other protein candidates including the lipid selectivity of PRDX1, we developed a complementary lipid-protein overlay assay with the aim to mimic the inner leaflet of the plasma membrane containing intracellular PtdIns lipids. Using recombinant active PI3Kγ, [γ-^{32}P]ATP, and lipid substrates in an *in vitro* kinase assay, we generated lipid micelles containing ^{32}P-labeled 3′-PtdIns ([^{32}P]PtdIns(X)P$_n$) and subsequently overlaid them onto membranes immobilized with the recombinant protein candidates (38). Precisely we used PtdIns(4)P or PtdIns(4,5)P$_2$ as a lipid source to generate ([^{32}P]PtdIns(3,4)P$_2$ and [^{32}P]PtdIns(3,4,5)P$_3$, respectively (see supplemental information). The quality of the radiolabeled [^{32}P]PtdIns(X)P$_n$ micelles was monitored by thin layer chromatography and is shown in the Fig. 4*B*. Validation of the micelle-based binding overlay was performed using recombinant PtdIns-binding proteins such as His-Akt/PKB, His-PDK1, and all protein kinase C isoforms (data not shown). During these tests, His-Akt/PKB full length and His-Akt/PKBΔPHD were used for each experiment as positive and negative controls, respectively. An example of lipid and protein selectivity is shown in Fig. 4*C* (*upper panels*) with the two different

radiolabeled 3′-[^{32}P]PtdIns(X)P$_n$ lipid products known to associate with Akt/PKB. To further validate the assay and confirm that binding of Akt/PKB to the lipids was due to PI3K products and not to a non-selective interaction originating from the micelles, we performed a lipid competition assay in which unlabeled PtdIns(3,4)P$_2$ (prepared with cold ATP) was used to dilute radiolabeled [^{32}P]PtdIns(3,4)P$_2$ products (Fig. 4*C*, *graph*, *lower panel*). Selectivity of Akt/PKB for [^{32}P]PtdIns(3,4)P$_2$ was confirmed by a decrease of its binding to Akt/PKB upon addition of increasing amounts of unlabeled, cold micelles containing PtdIns(3,4)P$_2$. Concomitantly this was further evidenced by the absence of lipid interaction when Akt/PKB lacking the PH domain (His-Akt/PKBΔPHD) was used as negative control.

We next tested the three candidate proteins with the lipid-protein assay. As depicted in Fig. 4*D*, we reproducibly confirmed the proper binding for PRDX1 with [^{32}P]PtdIns(3,4)P$_2$ and also identified a novel interaction for CAP1 (*upper panels*) but failed to show a reproducible interaction of the small GTPase Rab5c with the PI3K lipid product. Because recombinant proteins were generated with a GST tag, we further included recombinant GST as negative control in the overlay assay to confirm the absence of lipid interactions with GST (Fig. 4*D*, *lower panel*). Following the validation phase (Fig. 2) and the identification and confirmation of two potential novel PtdIns-binding proteins, we next asked whether the two-step LAC could be extended to study post-translationally modified PtdIns-regulated protein kinases.

Capture of Protein Kinase Activity from PtdIns-Protein Complexes—To first monitor global phosphorylation activity of the isolated lipid-bound protein complexes, two-step LAC eluates were used in an IVK assay using radiolabeled ATP in the absence of any substrate. Interestingly protein phosphorylation was increased when using 3′-phosphorylated PtdIns-(X)P$_n$ baits (Fig. 5*A*) indicating overall elevated protein kinase activity. To see whether this protein kinase activity correlated with isolation of activated Akt/PKB by the B-SS-PtdIns(X)P$_n$ baits during the two-step LAC, we next investigated B-SS-PtdIns(X)P$_n$ binding to endogenous Akt/PKB in its activated state in primary cells. As shown in Fig. 5*B* (*TCL*, *right panels*), growth factor stimulation (IGF1) induced Akt/PKB phosphorylation at residues Thr-308 and Ser-473 and increases its kinase activity (39). Two-step LAC analysis showed that only B-SS-PtdIns(3,4)P$_2$ and B-SS-PtdIns(3,4,5)P$_3$ eluates contained both phosphorylated and non-phosphorylated Akt/PKB as shown by specific antibodies against the two forms (Fig. 5*B*, *large panels*, *B-SS-PtdIns(X)P$_n$*). These results confirm that under these conditions neither of the two soluble lipid ligands interferes with the activity state of Akt/PKB kinase (40) during the two-step LAC.

We next asked whether protein kinases isolated by the two-step LAC could preserve their enzymatic activity for subsequent signal transduction studies. Fig. 5*C* demonstrates site-specific phosphorylation of recombinant GSK3

FIG. 4. **Confirmation of novel lipid-protein interactions by overlays.** *A*, protein-lipid overlay using nitrocellulose strips spotted with different lipids and further incubated with 0.5 μg of recombinant His-Akt/PKB or GST-PRDX1 as indicated. His-Akt/PKBΔPHD (lacking the amino-terminal PH domain) was used as negative control. *B*, thin layer chromatography analysis of [^{32}P]PtdIns(3,4)P$_2$ and [^{32}P]PtdIns(3,4,5)P$_3$ lipid products produced *in vitro* by incubation with recombinant GST-PI3Kγ (see supplemental information). *O*, origin. *C*, example of lipid-protein overlay showing the binding of His-Akt/PKB to *in vitro* reconstituted lipid micelles containing 2 μM [^{32}P]PtdIns(3,4)P$_2$ or [^{32}P]PtdIns(3,4,5)P$_3$ as indicated. Nitrocellulose membranes were spotted with 7.5 (*left*) and 3.75 (*right*) pmol of His-Akt/PKB and with 7.5 pmol of the control His-Akt/PKBΔPHD and further probed for protein load by immunoblot analysis using α-Akt/PKB antibodies as indicated below each overlay. *Lower panels*, recombinant His-Akt/PKB and His-Akt/PKBΔPHD (3.75 pmol) immobilized onto nitrocellulose membrane were incubated with 2 μM [^{32}P]PtdIns(3,4)P$_2$ micelles in the presence of increasing concentrations of cold PtdIns(3,4)P$_2$ (1.25–10 μM). [^{32}P]PtdIns(3,4)P$_2$ binding was digitized using a phosphorimaging device and further quantified by Quantity One software (density, counts/mm^2). Equal protein spotting of recombinant His-Akt/PKB was controlled by immunoblotting (α-*Akt/PKB*) as indicated. *D*, lipid-protein overlay of several recombinant proteins incubated with 2 μM [^{32}P]PtdIns(3,4)P$_2$ lipid micelles. Following each overlay, equal protein spotting was controlled by immunoblotting as indicated. *Upper panels*, GST-PRDX1 (7.5 and 3.75 pmol) and three additional GST-recombinant candidate proteins (3.75 pmol) tested with the respective negative (His-Akt/PKBΔPHD) and positive controls (His-Akt/PKB) using 7.5 pmol of recombinant proteins. *Lower panel*, GST and His-Akt/PKBΔPHD (3.75 pmol) used as additional negative controls, respectively. *LPA*, lysophosphatidic acid; *PS*, phosphatidylserine; *PA*, phosphatidic acid.

by Akt/PKB confirming preserved enzymatic activity. More specifically, serum-conditioned cells stimulated with IGF1 showed Akt/PKB-induced phosphorylation of recombinant GSK3 substrate at Ser-9 upon activation (*middle panel*, compare *lanes 9* and *10*). The resulting two-step LAC eluates (*upper panel*, *lanes 5–8*) displayed increased kinase activity in a stimulation-dependent manner (*middle panel*, compare *lanes 5–8*), further demonstrating that changes in catalytic activity of Akt/PKB can be captured by B-SS-PtdIns(3,4)P$_2$ or B-SS-PtdIns(3,4,5)P$_3$.

Finally to explore the possibility of using kinase activity as a readout to identify new lipid-complexed protein kinases pres-

ent in amounts probably too low for successful mass spectrometric identification, we performed an IGK assay using myelin basic protein as a generic serine/threonine kinase substrate. Corresponding two-step LAC eluates were separated by gel electrophoresis, and kinase enzymatic activity of the refolded proteins was visualized *in situ* by in-gel substrate phosphorylation (see supplemental information). The resulting autoradiograph displayed a ^{32}P phosphorylation pattern qualitatively indistinguishable between both B-SS-PtdIns(4,5)P$_2$ and B-SS-PtdIns(3,4)P$_2$ eluates; however, an increased intensity was noticed for the 3′-phosphorylated B-SS-PtdIns(3,4)P$_2$-bound protein complexes (see Supplemental Fig. 2). At least
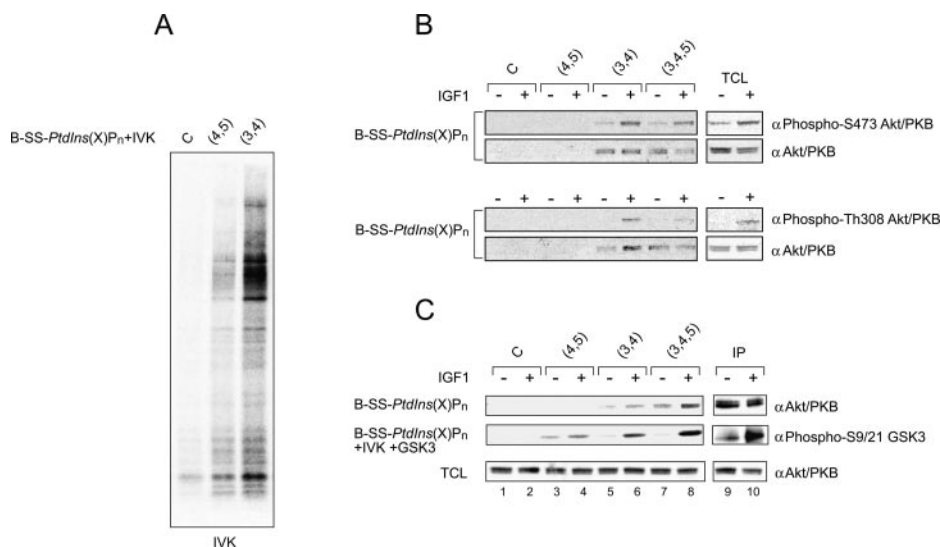
FIG. 5. **Signal transduction-based protein kinase activity.** *A*, elevated protein kinase activity of 3′-phosphorylated PtdIns(*X*)P$_n$-bound proteins. Phosphoinositide-bound protein complexes were isolated from primary BMDMs by two-step LAC using B-SS-PtdIns(3,4)P$_2$ and B-SS-PtdIns(4,5)P$_2$. Proteins were eluted from the lipid matrices by DTT and assayed for kinase activity. Following *in vitro* kinase reactions and SDS-PAGE, ^{32}P-labeled proteins were further digitized using a phosphorimaging device. *C*, streptavidin beads without addition of lipid baits as a control. *B*, Akt/PKB isolation by 3′-phosphorylated B-SS-PtdIns(*X*)P$_n$ does not interfere with its activity status. Capture of endogenous Akt/PKB from serum-conditioned BMDM cells in the presence or absence of IGF1 by B-SS-PtdIns(*X*)P$_n$ ligands as indicated is shown. Immunoblot analysis of two-step LAC eluates was performed using the indicated phosphospecific Akt/PKB antibodies. Immunoblots were stripped and reprobed with Akt/PKB antibodies as indicated (*α-Akt/PKB*, *lower panels*). *C*, streptavidin beads without addition of lipid baits as a control. Aliquots of the cell lysates prior to two-step LAC were used as a control for the overall phosphorylation status of IGF1-stimulated Akt/PKB (*TCL*, *right panels*). *C*, selective GSK3 substrate phosphorylation by active Akt/PKB. Endogenously expressed Akt/PKB was isolated by two-step LAC from HEK-293 cells using the indicated B-SS-PtdIns(*X*)P$_n$ lipid baits (*top panel*). Resulting eluates were further incubated with recombinant GSK3 for *in vitro* kinase reaction. Phosphorylation of GSK3 by 3′-phosphorylated PtdIns(*X*)P$_n$-purified Akt/PKB was detected by immunoblot using a phosphospecific (Ser(P)-9/21) GSK3 antibody recognizing the Akt/PKB phosphorylation site on GSK3 (*middle panel*). *C*, streptavidin beads without addition of soluble lipid ligands as a control. Immunoprecipitated (*IP*) Akt/PKB following *in vitro* kinase reaction is also shown as an IVK control (*right panel*). TCL controls (*bottom panel*) correspond to protein expression prior to two-step LAC or immunoprecipitation. *(4,5)*, B-SS-PtdIns(4,5)P$_2$; *(3,4)*, B-SS-PtdIns(3,4)P$_2$; *(3,4,5)*, B-SS-PtdIns(3,4,5)P$_3$.

seven bands representing potential B-SS-PtdIns(*X*)P$_n$-complexed protein kinases were reproducibly detected in each of the four independent experiments performed using primary macrophage cells.

<center>DISCUSSION</center>

Until now only a single large scale lipid affinity study has proved effective in isolating several novel proteins as evidenced by subsequent microsequence analysis and the characterization of ARAP3 as a novel PtdIns(3,4,5)P$_2$-regulated GTPase-activating protein (8). Although this is of high scientific relevance, this large scale approach is less applicable to situations in which only limited amounts of biological sample are available. Also in general, protein elution poses a challenge for the selective protein-bait recovery when conventional denaturing procedures are used. On the other hand, when salt is used instead, the direct consequence is that functional lipid-associated protein complexes dissociate, and information is lost for direct signal transduction studies. To circumvent these limitations, we undertook an approach combining the three-way intersection of (i) *biological chemistry* (cleavable lipid bait), (ii) *activity-based signal transduction*, (IVK/IGK assays), and (iii) *proteomics* (LAC and SEC-LAC) to isolate, interrogate, and identify the lipid signalosome of limited biological material.

Further to the absence of preparative sample scale-up, efficacy of the novel lipid baits was also demonstrated by subtle changes in the lipid moieties. Importantly the addition of a single phosphate group to the inositol ring of the cleavable bait confirmed proper lipid selectivity toward known interacting proteins. This was demonstrated by immunoblot analysis with several endogenous PI3K effector lipid-binding protein kinases as well as by silver-stained proteins (Supplemental Fig. 1). In addition to the high lipid-protein selectivity provided by the two-step LAC (Fig. 2), MS/MS protein identification was conducted after an analytical SEC performed prior to the two-step LAC and without sample scale-up. Although the SEC adds one more step for efficient protein identification, it allows for the targeting of a distinct set of protein complexes (size-based) while preserving their native state for subsequent signal transduction studies.

Following the identification of novel interactions by two-step LAC, additional evidence was necessary to prove direct lipid-protein interaction. This was exemplified by primary overlay assays for three candidate proteins selected from

Table III. We confirmed a direct interaction for two of them, namely PRDX1 and CAP1, but failed for the protein Rab5c; although from the cellular distribution and its link to PI3K, we anticipated an interesting potential for this candidate. Interestingly we also found that irrespectively of the positive and negative controls that were used the candidate proteins behaved differently depending on the primary overlay assay that was used. PRDX1 worked in both protein-lipid and lipid-protein assays, whereas for CAP1, proper binding was confirmed only when the micelle-based overlay was used instead. Although in theory one can argue about the reasons why one protein bound with one assay and why one did not, these differences illustrate the necessity to use various approaches prior to assigning or not assigning a given interaction. Notwithstanding these findings, the newly confirmed candidates will require further investigations to certify a direct novel lipid-protein association under physiological conditions.

Finally and to further extend the used of the two-step LAC, we integrated an additional dimension to the approach by subsequently capturing protein kinase activity. This is evidenced in Fig. 5 where we demonstrated the possible use of a lipid-binding protein kinase to phosphorylate its natural substrate, a procedure commonly used in signal transduction studies. In addition, the selective capture of protein kinase (or phosphatase) activity can help detect proteins that escape MS/MS protein identification. In fact, ATP-induced protein activity does not necessarily reflect the protein kinase expression level originally present in the two-step LAC eluates. Therefore, it is unlikely that they would have been identified by MS/MS analysis with so little biological sample. These results plus the selective isolation of known PtdIns(X)P$_n$-binding protein kinases (Fig. 2) only identified by immunoblotting provide evidence for the presence of a large set of direct and indirect lipid-associated protein kinases. Further assignment of their individual identity and direct effector proteins following receptor-ligand stimulation will require additional work (IGK assays using a broader range of selective protein kinase substrates or *in situ* isolation of the radiolabeled band followed by highly sensitive protein identification). As a matter of fact, the significant number of candidate proteins identified by single peptides in this study illustrates the challenge to apply chemical lipid for classical proteomics when such limited biological material is available. Also several low copy number proteins including small GTPases identified with several peptides were not implemented in the candidate list as the peptides did not allow differentiating between the families. Although these caveats can be compensated using more biological sample, we have demonstrated its feasibility and the value of the cleavable B-SS-PtdIns(X)P$_n$ when used in an integrative context with single, relevant primary cells. By exploring the interface between chemistry and biology, this integrated approach should help to establish a conceptual framework to extend our current thinking on lipid signal transduction and MS/MS protein identification, that is using a small molecule as bait to directly assess a novel protein function.

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