THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 279, No. 43, Issue of October 22, pp. 44763–44774, 2004
Printed in U.S.A.

Sphingosine Kinase 1 Is an Intracellular Effector of Phosphatidic Acid*

Received for publication, May 24, 2004, and in revised form, July 30, 2004
Published, JBC Papers in Press, August 13, 2004, DOI 10.1074/jbc.M405771200

Christine Delon‡, Maria Manifava‡, Eleanor Wood‡, Dawn Thompson§, Sonja Krugmann‡, Susan Pyne§, and Nicholas T. Ktistakis‡¶

From the ‡Department of Signalling, Babraham Institute, Cambridge CB2 4AT and the §Department of Physiology and Pharmacology, Strathclyde Institute for Biomedical Sciences, University of Strathclyde, Glasgow G4 0NR, United Kingdom

Sphingosine kinase 1 (SK1) phosphorylates sphingosine to generate sphingosine 1-phosphate (S1P). Because both substrate and product of the enzyme are potentially important signaling molecules, the regulation of SK1 is of considerable interest. We report that SK1, which is ordinarily a cytosolic enzyme, translocates *in vivo* and *in vitro* to membrane compartments enriched in phosphatidic acid (PA), the lipid product of phospholipase D. This translocation depends on direct interaction of SK1 with PA, because recombinant purified enzyme shows strong affinity for pure PA coupled to Affi-Gel. The SK1-PA interaction maps to the C terminus of SK1 and is independent of catalytic activity or of the diacylglycerol kinase-like domain of the enzyme. Thus SK1 constitutes a novel, physiologically relevant PA effector.

The potential function of PA[1] in signal transduction is not well established, and there is even debate about whether PA itself (or a downstream metabolite) is a signaling lipid (1). Whereas many cellular control pathways exhibit a stimulus-dependent elevation of PLD activity (arguing for a direct role of the lipid product of PLD-PA in these pathways (2)), historically, the search for classic protein effectors of PA has failed to yield many clear-cut examples where a specific response to elevated PA levels could be demonstrated directly.

Recent data from a number of laboratories are beginning to define some potential PA targets. These include kinases (*e.g.* Raf-1 (3, 4) and mammalian target of rapamycin (5)), phosphatases (*e.g.* protein-tyrosine phosphatase SHP-1 (6) and protein phosphatase-1 (7)), enzymes involved in lipid turnover (*e.g.* phosphatidylinositol-4-phosphate 5-kinase (8, 9) and phospholipase C (10)), as well as other enzymes (*e.g.* phosphodiesterase PDE4D3 (11, 12)). There are additional examples of proteins regulated by PA in combination with other lipids (*e.g.* protein kinase Cϵ (13) and p47^{phox} (14)). With the possible exception of Raf-1 and PKCϵ, regulation of the other proteins by PA has not been shown to result directly in membrane translocation *in vivo*. Raf-1 accumulates in endosomal membranes as a consequence of PA formation (4), whereas PKCϵ translocates to the plasma membrane in response to coordinated production of PA and diacylglycerol (13).

By phosphorylating sphingosine to generate S1P, SK1 regulates the levels of two important bioactive lipids and affects a number of cellular functions, including for example calcium mobilization, growth control, and cytoskeletal rearrangements among others (for a recent review see Ref. 15). We became interested in the possibility that SK1 may constitute a novel PA effector based on three types of evidence. SK1 activity is stimulated by acidic phospholipids, including PA (16), it is found up-regulated in immune cells following PLD activation (reviewed in Ref. 17), and the SK1 protein is activated by PKC (a PLD activator) and translocates to membranes following stimulation with phorbol esters (18).

In this work we show that SK1 is a PA effector and responds to PA formation by translocating to membranes in a manner analogous to other effectors of signaling lipids, most notably of the phosphoinositides. We propose that membrane translocation of SK1 is a direct consequence of the acute and localized formation of PA. This finding adds support to the idea that PA is a signaling lipid and provides novel avenues of investigation concerning the cellular functions of SK1.

MATERIALS AND METHODS

Plasmids—The wild type and the G81D catalytically dead (KD) mouse SK1 were cloned in pcDNA4/*myc*-His (Invitrogen). These and the N- and C-terminal SK1 fragments (residues 1–169 and 134–388, respectively) were also sub-cloned into pEGFP-C2 (Clontech) using appropriate primers and standard techniques. PLD1 was cloned in the pCMV3 vector as described previously (19).

Antibodies—The following antibodies were used in immunofluorescence and immunoblots: mouse monoclonal 9E10 against the myc epitope (prepared at Babraham); mouse monoclonal 6G5 against glyceraldehyde-3-phosphate dehydrogenase (Biogenesis); rabbit polyclonals raised against N- and C-terminal peptides of PLD (19); mouse monoclonal to phospholipase Cδ, a kind gift of Drs. Matilda Katan and Matthew Jones (Institute of Cancer Research, London); rabbit polyclonal against GFP (Molecular Probes); rabbit polyclonal against the medial Golgi marker mannosidase II (Dr. Kelley Moremen, University of Georgia); mouse monoclonal against early endosome antigen 1 (EEA1, BD Transduction Laboratories). Secondary fluorescein-, rhodamine-, or horseradish peroxidase-conjugated antibodies were from Jackson ImmunoResearch Laboratories. Biotin dextran was detected with Cy3-conjugated streptavidin (Jackson ImmunoResearch). SPE7 IgE antibodies against DNP used to prime RBL-2H3 cells were from Sigma.

Cell Culture—COS-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), and 1% penicillin and streptomycin (Invitrogen). CHO_{tet} cells were maintained in nutrient mixture F-12 (Ham's) with L-glutamine (Invitrogen) with 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were stably transfected with PLD1 using the Tet-on system (Stratagene) and were a kind of gift of Dr. Michael

* This work was supported by the Biotechnology and Biological Sciences and Research Council (to C. D., M. M., E. W., and N. T. K.), the Medical Research Council (to D. T. and S. P.), and Cancer Research UK (to S. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 44-1223-496-323; Fax: 44-1223-496-043; E-mail: nicholas.ktistakis@bbsrc.ac.uk.

[1] The abbreviations used are: PA, phosphatidic acid; PLD, phospholipase D; PKC, protein kinase C; CMV, cytomegalovirus; GFP, green fluorescent protein; DNP, dinitrophenyl; DMEM, Dulbecco's modified Eagle's medium; CHO, Chinese hamster ovary cells; S1P, sphingosine 1-phosphate; PC, phosphatidylcholine; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; SK1, sphingosine kinase 1.

Downloaded from http://www.jbc.org/ at Babraham Institute on July 17, 2015

Frohman (State University of New York, Stony Brook, NY). PLD expression was induced by incubation with 1 μM doxycycline in normal medium for 18 h prior to analysis. RBL-2H3 cells were a kind gift of Dr. Shamshad Cockcroft (University College London, London, UK) and were grown in DMEM and 10% heat-inactivated (56 °C for 32 min) in fetal bovine serum and 2 mM glutamine. All cells were incubated in a humidified atmosphere at 37 °C in 5% CO_2 and grown on Nunc plastic ware or on glass coverslips for immunofluorescence. COS-7 cells were transfected using DEAE-dextran as described before (19). All other cells were transfected by electroporation.

Microscopy—Cells for immunofluorescence were grown on glass coverslips and fixed in 3.7% formaldehyde in 200 mM Hepes, pH 7.0. They were permeabilized in 0.25% Nonidet P-40 in NET gel (150 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl, pH 7.4, 0.05% Nonidet P-40, 0.25% gelatin, and 0.02% sodium azide) and blocked with NET gel. Cells were stained with the appropriate primary and secondary antibodies in NET gel, and coverslips were mounted on a drop of Aqua Poly Mount (Polysciences Inc.). Samples were visualized with a Zeiss Axiophot 2 fluorescence microscope using a ×60 oil immersion lens or a ×40 lens. Photographs were taken with a SPOT digital camera (Diagnostic Instruments Inc.) and supplied software.

Translocation of SK1 to Membrane Fractions in Vitro—CHO-K1 light membranes were isolated from a 0.8–1.2 M sucrose interface as described before (20). COS-7 cytosol from cells transfected with SK1 was prepared as described (21) and was diluted 1/25 in COS-7 cytosol from untransfected cells so that the signal from the input fraction would fall within the linear range of the ECL detection. This cytosol mixture was pre-cleared by centrifugation at 14,000 × *g* at 4 °C for 5 min to remove any insoluble protein and was used in the assay. For a binding assay, membranes were diluted 1/5 in Buffer B (25 mM KCl, 2.5 mM $MgSO_4$, 1 mM dithiothreitol, 0.2 M sucrose, 25 mM Hepes, pH 7.2) and added to the cytosol mixture into siliconized microcentrifuge tubes. The ratio of cytosol to membranes was ~1:7 in a total volume of 200 μl. In addition to membranes and cytosol, binding reactions contained 1 mM EGTA, 1.2 mM $CaCl_2$, 2 mM ATP, and 2.5 units of *Streptomyces chromofuscus* (Sigma catalogue number P8023) or *Streptomyces* sp. (Sigma catalogue number P4912) PLD as indicated. The samples were mixed gently and incubated at 37 °C for 15 min. At the end of incubation the samples were centrifuged at 14,000 × *g* at 4 °C for 10 min, the supernatant was carefully aspirated, and the membrane pellets were resuspended in 15 μl of Laemmli sample buffer for analysis by SDS-PAGE.

Measurement of SK1 Activity—Light membranes from CHO cells not expressing myc-SK1 were incubated with cytosol from COS cells expressing myc-tagged SK1 in the presence of calcium, PLD, or calcium/PLD as required and harvested by centrifugation. Membranes were resuspended in SK assay buffer (20 mM Tris-HCl, pH 7.4, 20% (v/v) glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 40 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM 4-deoxypyridoxine, 10 μg/ml aprotinin, leupeptin, and soybean trypsin inhibitor) to ~0.6 mg of protein/ml. Aliquots of membranes were added to glass vials in which sphingosine (0 or 10 nmol) and phosphatidylserine (50 nmol) was presented as mixed micelles using Triton X-100 (final concentration 0.063%). Reactions were initiated by the addition of ATP (1 μCi, 40Ci/mmol) in 10 mM $MgCl_2$ and incubated for 60 min at 37 °C. Resulting [^{32}P]S1P was extracted using butanol, which was washed twice using 2 M KCl before quantification of radioactivity. Analysis of the radioactive butanol-soluble material by TLC confirmed the presence of a single product, which co-migrated with an S1P standard.

Binding of SK1 to Lipid-coupled Beads—These experiments were done essentially as described before (22) using lipid-coupled beads whose detailed synthesis has recently been described (23). COS-7 cells transiently transfected with the appropriate constructs were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM EDTA, 0.6 mM phenylmethylsulfonyl fluoride, 1 μg/ml trypsin inhibitor, and 0.5% Nonidet P-40) and centrifuged at 14,000 × *g* to remove debris. The supernatant, with ~500–600 μg/ml protein, was collected and diluted 1:1 in lysis buffer to give the lysate. 380 μl of lysate was incubated with 30 μl of a 10% (v/v) suspension of PA beads in IP buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, and 0.1% Nonidet P-40) in a 500-μl microcentrifuge tube on ice. A few air bubbles were included to facilitate mixing of the beads. The tubes were put in a rotator at 4 °C for 2 h. At the end of binding, beads were quickly washed three times with IP buffer and resuspended in Laemmli sample buffer for SDS-PAGE. For experiments using soluble lipid as competitor during the binding reaction, lipids in chloroform were dried under a stream of nitrogen and resuspended by sonication in lysis buffer. The appropriate amount was added to the cleared COS-7 lysates for 15 min on ice before addition of lipid-coupled beads.

Binding of SK1 to Liposomes—Lipid stocks were kept at 10 mg/ml in chloroform. To produce 1 ml of liposomes, 32 μl of PC, 32 μl of phosphatidylethanolamine, and 10 μl of phosphatidylserine were mixed, dried under a stream of nitrogen, and incubated with 1 ml of HK buffer (40 mM Hepes, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 1 mM EGTA, 2.5 mM $MgCl_2$) for 4 h at 4 °C. At the end of this incubation liposomes were produced by vortexing for 3 min. Each binding reaction contained 120 μl of liposomes, the indicated units of bacterial PLD, plus SK1 either purified or as part of a cytosolic fraction, to give a total volume of 150 μl. Binding was allowed at 30 °C for 15 min followed by centrifugation in a microcentrifuge and careful aspiration of the supernatant. To measure PC hydrolysis in this system, 0.5 μCi of [^{14}C]PC was included in the lipid mixture before drying. At the end of the binding reaction, lipids were extracted with methanol:chloroform essentially according to Bligh and Dyer (see Ref. 24). Lipids were dried, resuspended in 30 μl of chloroform, loaded onto TLC plates (Whatman LK6DF silica gel 60A, 250 μm), and separated in a solvent system containing chloroform:methanol:acetic acid:acetone:water (10:2:2:4:1).

Microinjections with Bacterial PLD—COS cells were transiently transfected with the appropriate GFP construct and seeded onto glass coverslips or on glass bottomed dishes (World Precision Instruments) for 40 h. 3 units/μl *Streptomyces* sp. PLD with 2 mg/ml biotin dextran (M_r 3000, Molecular Probes) were microinjected using an Eppendorf Tranjector coupled to a micromanipulator. For immunofluorescence, 100–150 cells on each coverslip were microinjected over a period of 15 min in a temperature- and CO_2-controlled chamber, fixed, and stained with Streptavidin-Cy3. For live imaging, two or three cells were microinjected and imaged at 15- or 30-s intervals for 10–20 min. Imaging was carried out with a Zeiss Axiocam camera and Axiovision software. In all imaging experiments, a subset of cells was microinjected with vehicle alone plus dextran. This treatment did not have any effects on the movement of SK1.

Measurement of PLD Activity in Vivo—RBL-2H3 cells were labeled and stimulated as follows. [^{3}H]Palmitate in ethanol was dried under a stream of nitrogen and resuspended in dialyzed serum. Cells to be labeled were washed with serum-free DMEM and incubated for 16 h in DMEM containing 10% dialyzed serum, 2 mM L-glutamine, and 60 μCi/ml palmitate. 25 ng/ml anti-DNP IgE was included to sensitize the FcεRI. Subsequently, medium was replaced with fresh DMEM containing 10% serum, 2 mM L-glutamine, and, as indicated, 25 μg/ml DNP conjugated with bovine serum albumin and 1% butanol. After 30 min of incubation at 37 °C, lipids were extracted and resolved as described above. Plates were then dried, sprayed with EN^{3}HANCE (DuPont), and exposed to film at −80 °C.

Expression of SK1 in RBL-2H3 Cells—RBL-2H3 cells were transfected with 30 μg of pEGFP-C2-SK1 by electroporation using 0.4-cm gap electroporation cuvettes (Bio-Rad) at 0.27 V and 960 microfarads with a time constant approaching 30 ms (Bio-Rad GenePulser™). Cells were seeded on glass coverslips to be ~70% confluent after 24 h. After 8 h cells were primed by the addition of fresh medium containing 25 ng/ml DNP IgE for 16 h to sensitize the FcεRI. Cells were stimulated by the addition of fresh medium containing 25 μg/ml DNP-bovine serum albumin for 30 min and then fixed and stained with antibodies to myc. For experiments done in the presence of alcohols, medium containing either butanol or *sec*-butanol was used during the 30-min stimulation.

Reproducibility of Data—All data shown have been reproduced at least three times. Experiments with SK1 activity and microinjections were done such that the person measuring activity or microinjecting cells did not know the identity of the samples until after the experiment was finished.

RESULTS

Co-localization of SK1 with PLD1—Co-expression of SK1 with PLD1 under basal conditions resulted in some co-localization of the two proteins, primarily on perinuclear vesicular structures (Fig. 1*A*). This was consistent with the possibility that SK1 becomes enriched in membranes containing increased amounts of PA as a result of PLD1 overexpression. To examine this possibility further we expressed SK1 in CHO cells that were designed to overexpress PLD1 inducibly (Fig. 1*B*). Without PLD1 expression, SK1 was localized mainly in the cytosol and in some perinuclear regions (Fig. 1*B*, *upper three panels*). Upon PLD1 induction followed by stimulation with phorbol ester (*i.e.* under conditions of maximal PLD1-derived PA formation), SK1 accumulated in a perinuclear region that co-localized very well with PLD1 (Fig. 1*B*, *lower three panels*). In

Downloaded from http://www.jbc.org/ at Babraham Institute on July 17, 2015

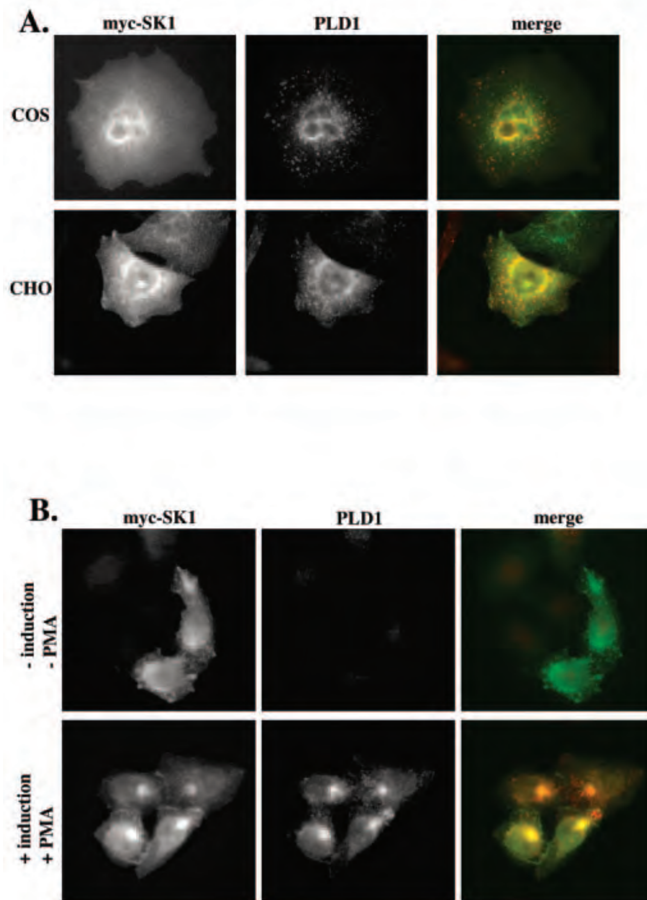


FIG. 1. **Co-localization of SK1 with PLD1 under basal and stimulated conditions.** *A*, plasmids expressing myc-tagged SK1 and untagged PLD1 were co-expressed in COS or CHO cells as indicated. Cells were fixed and stained with antibodies to the myc tag (mouse) and to PLD1 (rabbit) followed by appropriate secondary antibodies. *B*, CHO cells expressing PLD1 inducibly were transiently transfected with myc-tagged SK1. Cells were either left untreated, or induced for 18 h for PLD1 expression followed by 30-min stimulation with 100 nM phorbol 12-myristate 13-acetate (*PMA*) as indicated. At the end of treatments cells were fixed and stained as in *A*.

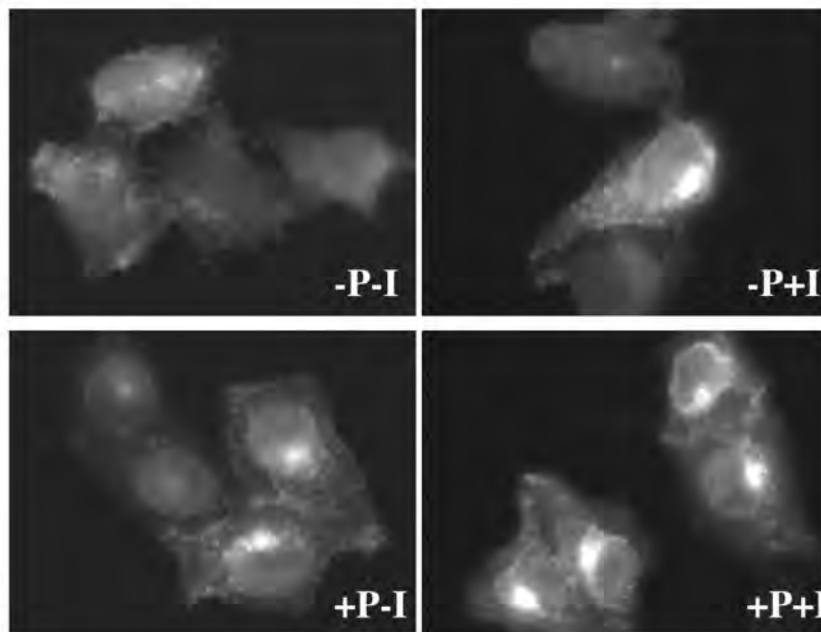
addition to the perinuclear accumulation, some SK1 also became enriched on the plasma membrane. In another set of experiments we examined the relative contributions of PLD1 induction and phorbol ester stimulation on the perinuclear accumulation of SK1 (Fig. 2*A*). Maximal SK1 translocation required both PLD1 induction and phorbol ester stimulation, although we frequently saw perinuclear accumulation of SK1 only with PLD1 induction or with phorbol ester stimulation (Fig. 2*B*). Further experiments determined that the perinuclear locus of SK1 co-localized partially both with the endosomal protein EEA1 and with the Golgi mannosidase 2 (data not shown). Finally, by co-immunoprecipitation experiments under a variety of conditions we obtained no evidence that SK1 and PLD1 interact directly (data not shown).

SK1 Translocates to PA-enriched Membranes and Is Enzymatically Active—To test directly the idea that SK1 binds to PA-enriched membranes we established an *in vitro* system. COS cells were transfected with plasmids expressing myc-tagged SK1, and a membrane-free cytosolic fraction was isolated. At the same time, a cytosol-free light membrane fraction was isolated from CHO cells that did not express myc-tagged SK1. The cytosolic and membrane fractions were then mixed in the presence of various added components at 37 °C, and the membranes were re-isolated by centrifugation and analyzed for SK1 binding by SDS-PAGE and immunoblots (Fig. 3*A*). To generate high levels of PA on the membranes, we used purified bacterial PLD from *S. chromofuscus*, an enzyme that hydrolyzes PC to generate PA in the presence of micromolar amounts of calcium (25). Simple addition of SK1 cytosol to membranes resulted in minimal translocation (Fig. 3*A*, *lane 2*). Similarly, the levels of SK1 found on membranes under most conditions of varying calcium and ATP were low. However, a very dramatic enhancement in the amount of translocated SK1 was obtained under conditions of maximal PLD activity (Fig. 3*A*, *lanes 7* and *8*). Without membranes, only a small amount of SK1 was found in the pellet fraction under fully activated conditions (Fig. 3*A*, *lane 9*). We then asked whether the SK1 that translocated to membranes under these conditions was still enzymatically active. At the end of a scaled-up binding experiment, the membranes were re-isolated without detergent and were assayed for SK1 activity (Fig. 3*B*). We found that SK1 translocation as shown by immunoblots of the same experiment (Fig. 3*B*, *inset*) was accompanied by an 8- to 10-fold increase in SK1 activity. We note that the -fold translocation of the protein paralleled the -fold increase in catalytic activity (both ~6- to 8-fold), suggesting that PA probably influences this pathway only at the level of membrane translocation.

Selective Binding of SK1 to Pure PA—Translocation of SK1 to PA enriched membranes as shown above could be indirect. To examine this possibility we assayed binding of recombinant SK1 to various lipids coupled covalently to Affi-Gel. These reagents have proven extremely useful in identification and characterization of various lipid binding proteins (22, 23, 26). Incubation of COS cell lysates enriched for SK1 with a variety of lipid-coupled beads showed specific binding of SK1 to PA (Fig. 4*A*). Of all the other lipids tested, only phosphatidylinositol 3-phosphate showed weak (less than a tenth of that seen for PA) binding. To examine the possibility that binding to PA involved the kinase activity of SK1, we repeated the binding experiment but using a catalytically compromised mutant of SK1 (Fig. 4*B*). For both wild type and mutant SK1, we saw strong and equivalent binding to PA and weaker binding to phosphatidylinositol 3-phosphate. To provide evidence for the specificity of the SK1-PA interaction, we carried out additional binding experiments to PA-coupled beads in the presence of various competitor lipids. Preincubation of SK1 lysates with increasing amounts of soluble PA (either dioctanoyl, C:8 or dipalmitoyl, C:16, which is the same species used for coupling) reduced significantly binding of SK1 to the PA-coupled beads (Fig. 4*C*). As we have reported before, the shorter chain analogue of PA was more effective for competition (22). Significantly, neither soluble sphingosine (the substrate of SK1) nor soluble phosphatidylserine (an acidic phospholipid capable of activating SK1 *in vitro* (16)) were able to compete for the binding of SK1 to PA (Fig. 4*D*).

The above data establish that SK1 has specific affinity for PA but do not rule out the possibility that this affinity is indirect, perhaps via an associated protein. To examine this possibility we purified recombinant SK1 from COS cells transfected with plasmids encoding myc- and His-tagged SK1. In this preparation, SK1 was the major protein as shown by Coomassie staining (data not shown). Using this highly enriched SK1 sample, we showed that binding to immobilized PA was still strong (Fig. 5*A*), at the expense of some additional bands that copurify with the protein but were much reduced from the PA-enriched fraction (Fig. 5*A*, *bands marked with asterisks*). We then examined binding of recombinant purified SK1 to pure liposomes that were enriched for PA by incubation with bacterial PLD. For these experiments we used a preparation of *Streptomyces* sp. PLD that does not require calcium for activity

Downloaded from http://www.jbc.org/ at Babraham Institute on July 17, 2015



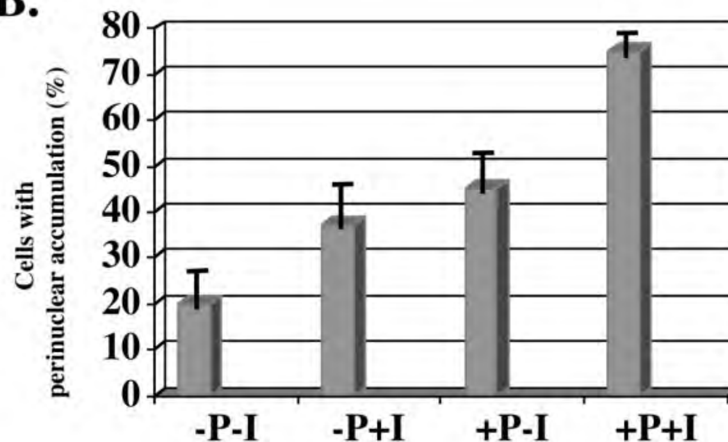


FIG. 2. **Translocation of SK1 to perinuclear membranes after PLD1 activation.** *A*, CHO cells expressing PLD1 inducibly were transiently transfected with myc-tagged SK1 and treated in various ways. In one set, cells were induced for PLD1 expression for 18 h followed by 30-min stimulation with 200 nM phorbol 12-myristate 13-acetate (*+P+I*). In the other three sets, induction (*I*) and phorbol 12-myristate 13-acetate treatment (*P*) were varied as indicated. At the end of treatments, cells were fixed and stained for SK1 using anti-myc antibodies. *B*, cells from three independent experiments as shown in *A* were counted to provide an estimate of the extent of perinuclear accumulation of SK1 following various treatments. On average, 300 transfected cells were counted.

Downloaded from http://www.jbc.org/ at Babraham Institute on July 17, 2015

(see also Fig. 7*D*) to avoid potential artifacts caused by calcium during liposome preparation. Translocation of SK1 to these defined liposomes was dependent on active PLD (Fig. 5*B*), and it occurred at the same concentration range of the enzyme that gave measurable PA formation (Fig. 5*C*). We have done additional experiments varying the base composition of liposomes (including ones made with PC only) but have never obtained translocation of SK1 that was not dependent on PLD activity. In addition, SK1 present in a cytosolic fraction translocated to the PLD-treated liposomes with similar efficiency as the purified protein, indicating that the SK1-PA interaction is direct (data not shown).

Binding to PA Does Not Involve the Diacylglycerol Kinase-like Domain—The amino acid sequence of SK1 offers little information on functional domains similar to other proteins, with the exception of a diacylglycerol kinase-like region at the N terminus (27). This region shares significant similarities with diacylglycerol kinases from other species and contains the residues that mediate catalytic activity of SK1 (27, 28). To determine if this region is involved in PA recognition we created a full-length SK1 and two fragments of SK1 tagged with the green fluorescent protein (GFP) as a reporter (Fig. 6*A*). The full-length GFP-SK1 bound to PA as well as the myc-tagged version (Fig. 6*B* in comparison to Fig. 4*A*). Interestingly, among the two fragments examined, the majority of PA binding mapped to the C-terminal half (Fig. 6, *B* and *C*). We concluded from these results that SK1 binding to PA is independent of the diacylglycerol kinase-like domain and involves sites at the C-terminal half of the protein. Further mutagenesis of this region has indicated that PA recognition probably involves more than one sub-domain, and additional work is in progress to identify precisely the PA binding determinant.

Dramatic Redistribution of SK1 in Vivo after Rapid Elevation of PA Levels—The data described thus far suggest that SK1 would respond very effectively to elevation of cellular PA levels. To test this directly we sought to identify ways to produce a very rapid wave of PA in intact cells. Bacterial PLDs are again an obvious choice based on their robust activity and their non-reliance on cellular protein co-factors. However these enzymes must also be able to function in the known intracellular

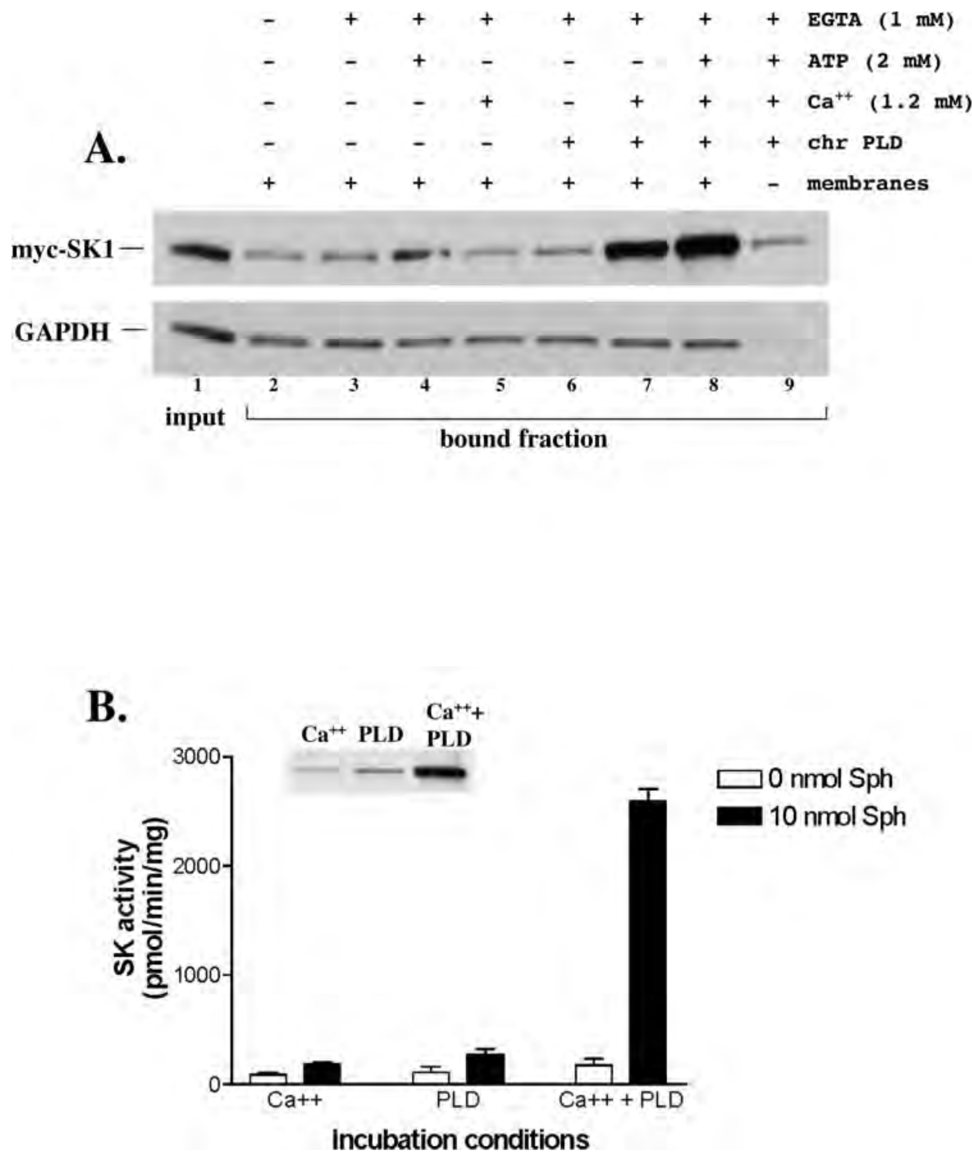


FIG. 3. **PLD-dependent translocation of active SK1 to membranes.** *A*, cytosol from COS cells expressing myc-tagged SK1 (*input*) was incubated with a light membrane fraction from CHO cells not expressing myc-SK1. Incubations contained additional components as indicated and were for 15 min at 37 °C. At the end of incubation, membranes were collected by centrifugation, and their SK1 content was analyzed by SDS-PAGE and immunoblotting. Notice that addition of active PLD (*i.e.* in the presence of calcium) greatly stimulated translocation of SK1 to the membrane fraction. The blot was also probed for glyceraldehyde-3-phosphate dehydrogenase (which is an abundant and slightly sticky protein) to give an indication of levels of nonspecific binding to membranes. *B*, membranes and cytosol as in *A* were scaled up 10 times to produce sufficient material for assaying SK1 activity after translocation. Only three conditions, varying calcium and PLD as indicated, were used. A portion of the membranes after translocation was analyzed by SDS-PAGE and immunoblotting (*inset gel*). The rest was analyzed in duplicate for SK1 activity using sphingosine and [γ-^{32}P]ATP. SK activity data are combined from replicate experiments.

conditions of pH, ion concentration, etc. For example the *S. chromofuscus* PLD will not work effectively in the low calcium environment of the cytosol, whereas other PLD enzymes require low pH for activity. However, the *Streptomyces* sp. PLD used in the liposome experiments (Fig. 5, *C* and *D*) did not require calcium for activity. Indeed, side by side comparison of this enzyme with the one from *S. chromofuscus* in the *in vitro* translocation assay for SK1 showed that calcium was not required for translocation (Fig. 7*D*, compare *lanes 4* and *6*), and in fact it was slightly inhibitory (Fig. 7*D*, compare *lanes 6* and *7*). This enzyme was therefore microinjected in intact cells expressing GFP-SK1 to assess translocation. In all cells microinjected with PLD, the GFP-SK1 distribution was rapidly changed from a cytosolic pattern to a reticular one that contained a large proportion of perinuclear staining (Fig. 7*A*). Additional experiments were performed with the N- or C-terminal fragments of SK1 tagged with GFP, or with GFP on its own as a negative control. Only the C-terminal fragment, which was shown to bind to PA (Fig. 6), showed PLD-dependent translocation (Fig. 7*B*). The N-terminal fragment and the GFP control did not change their localization upon PLD microinjection (Fig. 7*B*). To gain a better understanding of the kinetics of translocation, we microinjected GFP-SK1-expressing cells with PLD and followed the SK1 movement by live imaging. Within 3 min of microinjection, SK1 started to concentrate in a perinuclear region and also redistributed to a reticular network very reminiscent of the endoplasmic reticulum. Seven minutes after microinjection were sufficient for the complete redistribution of SK1 to membranes (Fig. 7*C*). The C-terminal fragment of SK1 showed a similar translocation but with slightly slower kinetics, whereas the GFP control did not show any translocation (data not shown).

Re-distribution of SK1 in Mast Cells after PLD Activation as a Result of Fc Receptor Cross-linking—The data using PLD

Downloaded from http://www.jbc.org/ at Babraham Institute on July 17, 2015

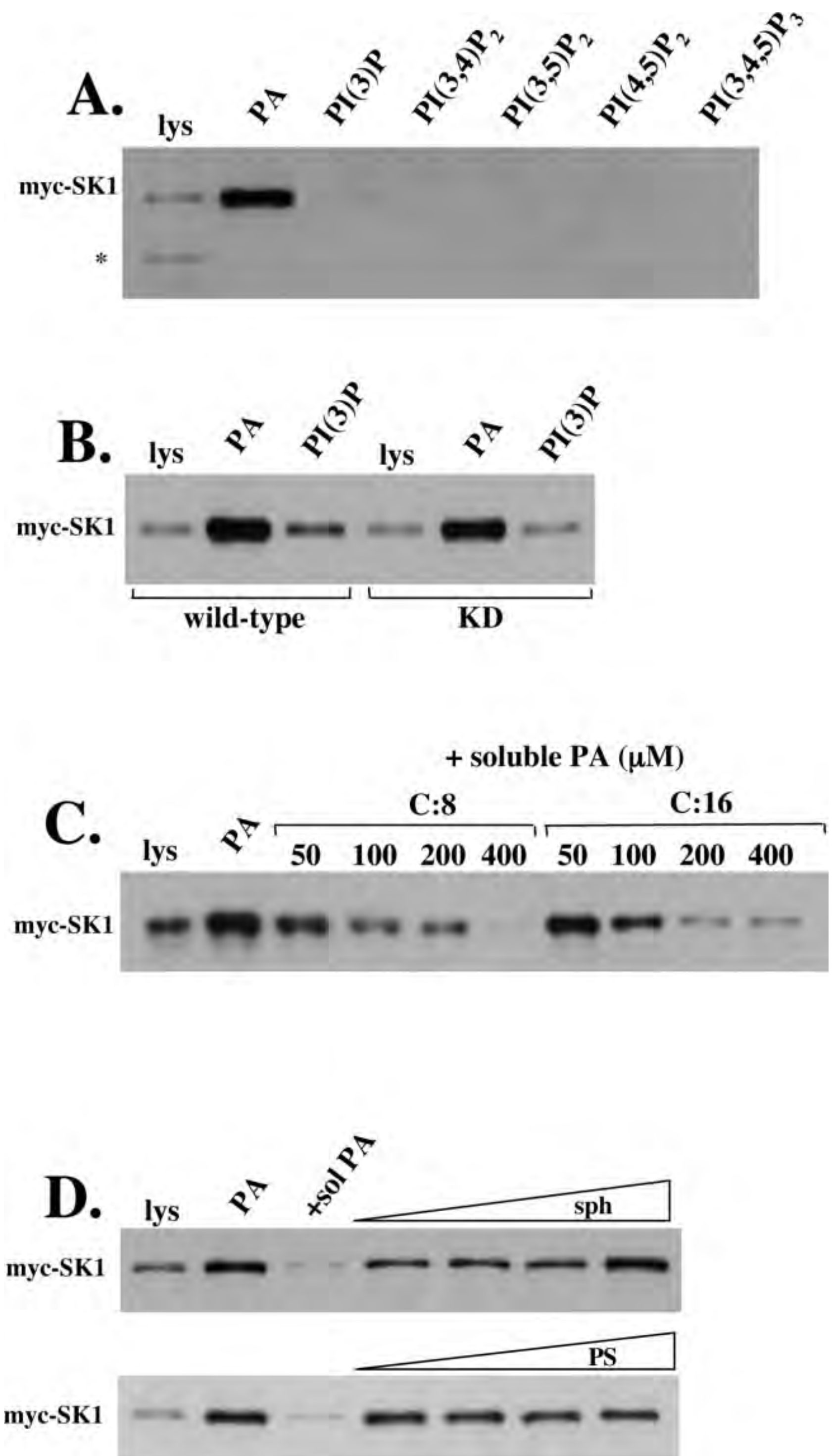


FIG. 4. **Binding of SK1 to pure PA.** *A*, lysates from COS cells expressing myc-tagged SK1 were mixed with Affi-Gel beads covalently coupled to the lipids shown. At the end of binding, the beads were washed extensively, and bound material was eluted and resolved by SDS-PAGE. SK1 was detected after immunoblotting via the myc epitope. Note the specific binding of SK1 to PA-coupled beads. The *band* labeled with an *asterisk* is recognized by the myc antibody nonspecifically. "*lys*" indicates total lysate loaded at 5% of input. *B*, experiment as in *A* but comparing binding to PA or phosphatidylinositol 3-phosphate of wild type or catalytically inactive (*KD*: G81D mutation) SK1. *C*, binding of SK1 to immobilized PA was carried out in the presence of the indicated amounts of soluble PA, either dioctanoyl, C:8 or dipalmitoyl, C:16. Notice that both PA species are capable of reducing binding of SK1 to PA beads. *D*, competition using sphingosine (*upper panel*) or phosphatidylserine (*lower panel*) at the same concentration range shown for PA above (50, 100, 200, and 400 μM). In both experiments, C:8 PA at 400 μM was included as a positive control for inhibition of binding.

microinjections provide a measure of the affinity of SK1 for PA in intact cells but do not actually define the site(s) of SK1 translocation upon physiologically relevant PA formation. To address this question we used the rat mast cell line RBL-2H3. Primary mast cells exhibit a strong receptor-dependent PLD activation (29), which is recapitulated in the established RBL-2H3 cell line (30). For these experiments we used a sub-clone of RBL-2H3 cells, which shows a good elevation of PLD activity upon Fc receptor cross-linking (31). RBL-2H3 cells were transfected with GFP-SK1 and incubated with IgE overnight. Under these conditions, the majority of GFP-SK1 was found in a cytosolic distribution (Fig. 8*A*, *upper panel*). Upon antigen addition and receptor cross-linking, a substantial portion of GFP-SK1 translocated to plasma membrane patches as well as to a perinuclear area (Fig. 8*A*, *lower panel*). Under exactly the same conditions, we saw a 5-fold increase in the amount of PLD-derived lipid product (phosphatidylbutanol) upon antigen stimulation (Fig. 8*B*, compare lane labeled "+*but*-*Ag*" with lane labeled "+*but*+*Ag*"). To test the idea that translocation of SK1 was in response to PLD activation, cells were treated with primary or secondary alcohols during stimulation. Primary alcohols inhibit formation of PA, whereas secondary alcohols do not under these conditions (31). We saw that 1% butanol blocked antigen-dependent SK1 translocation, whereas 1% *sec*-butanol was without effect (Fig. 8*C*), arguing that translocation depends on PA formation.

Downloaded from http://www.jbc.org/ at Babraham Institute on July 17, 2015

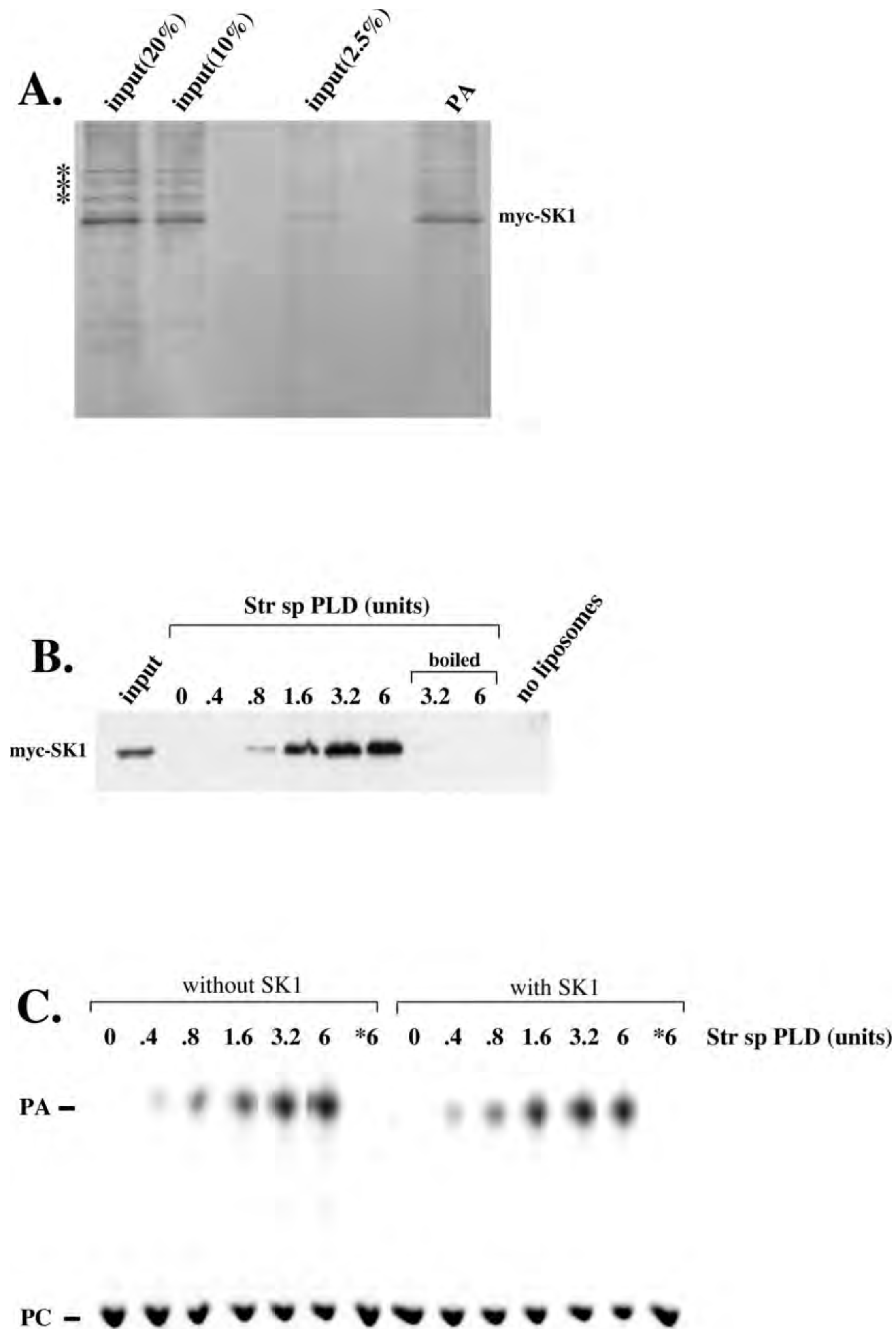


FIG. 5. **Purified recombinant SK1 binds to PA.** *A*, purified SK1 (following expression in COS cells and affinity purification using the His tag) was incubated with PA-coupled beads. After extensive washes, the bound fraction was resolved by SDS-PAGE and stained with silver. The *bands* labeled with *asterisks* contaminate the starting population but are substantially reduced in the bound fraction. *B*, purified SK1 as shown in *A* was incubated with liposomes of defined composition, in the presence of the indicated amounts of *Streptomyces* sp. PLD. Following binding at 30 °C, the liposomes were re-isolated by centrifugation and analyzed for bound SK1 by SDS-PAGE and immunoblots. Note that boiled PLD (for 5 min at 95 °C) at the two highest concentrations used for the normal binding does not induce any SK1 translocation. *C*, liposomes as in *B* but containing [^{14}C]PC were incubated with the indicated amounts of PLD in the presence or absence of recombinant SK1 as shown. At the end of incubation as in *B*, lipids were extracted and analyzed by TLC. *, boiled PLD as used in *B*.

Downloaded from http://www.jbc.org/ at Babraham Institute on July 17, 2015

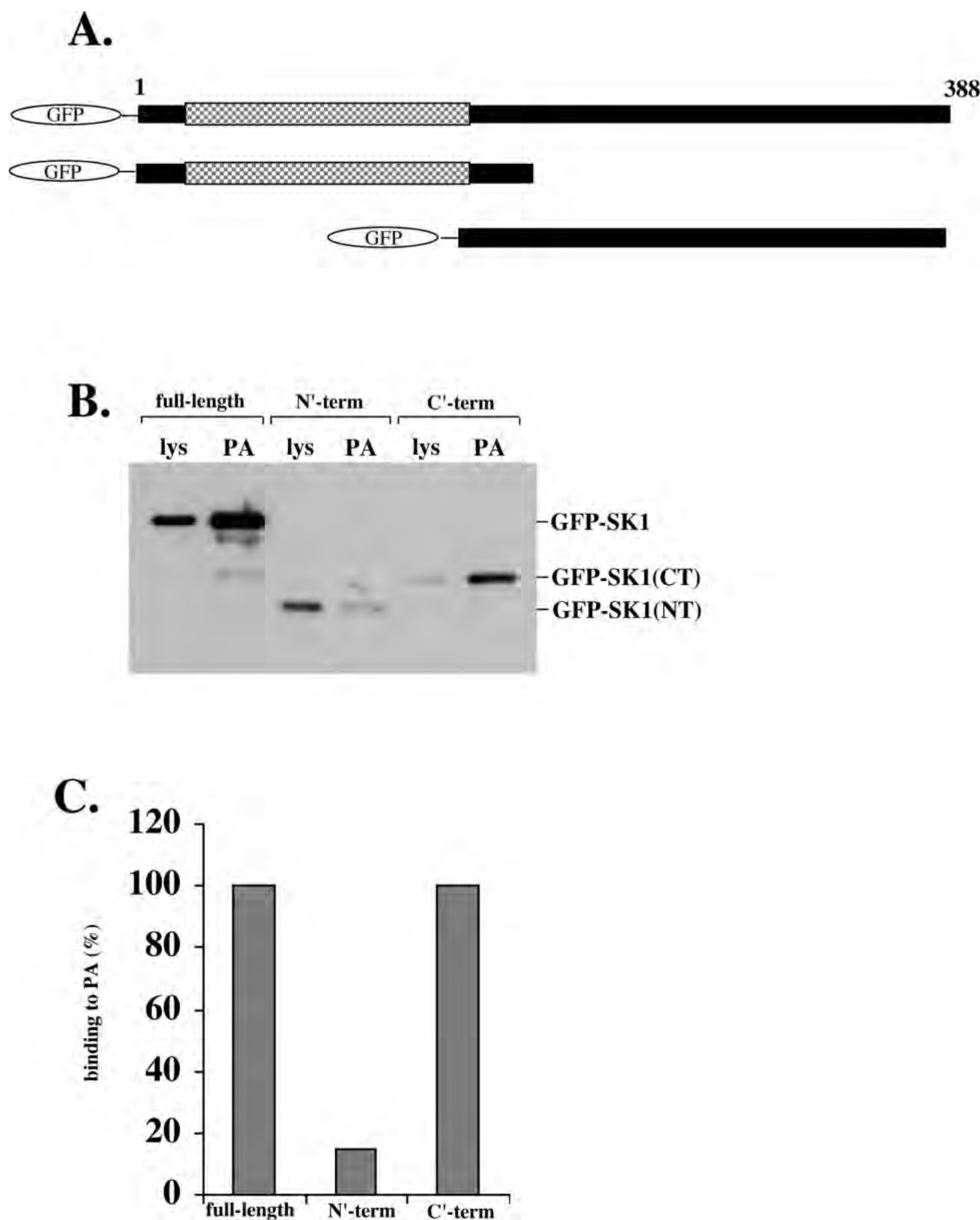


FIG. 6. **Interaction of SK1 with PA maps to the C′ terminus and does not involve the diacylglycerol kinase-like domain.** *A*, SK1 and N- and C-terminal fragments were tagged with GFP at the N′ terminus as shown. The *shaded region* represents the diacylglycerol kinase-like domain of SK1 and is drawn to scale (amino acids 25–127). The N-terminal fragment encompasses amino acids 1–169 and C-terminal fragment amino acids 134–388 of SK1. *B*, lysates from COS cells expressing full-length or truncated versions of GFP-tagged SK1 as shown in *A* were mixed with PA-coupled beads and allowed to bind. At the end of binding, the beads were washed extensively, and bound material was eluted and resolved by SDS-PAGE. SK1 was detected after immunoblotting via the GFP epitope. *C*, quantification of the blot shown in *B*. The relative binding of wild type GFP-SK1 to PA (the ratio of the band between lysate and PA fraction) was set to 100%. This was compared with the ratios of the N- and C-terminal fragments.

DISCUSSION

Based on our initial observations that SK1 co-localizes with PLD1, especially under conditions of PLD1 activation, and on the known involvement of SK1 in immune cell signaling downstream of PLD, we explored the possibility that SK1 is a PA effector. We provide complementary evidence from *in vivo* and

Downloaded from http://www.jbc.org/ at Babraham Institute on July 17, 2015

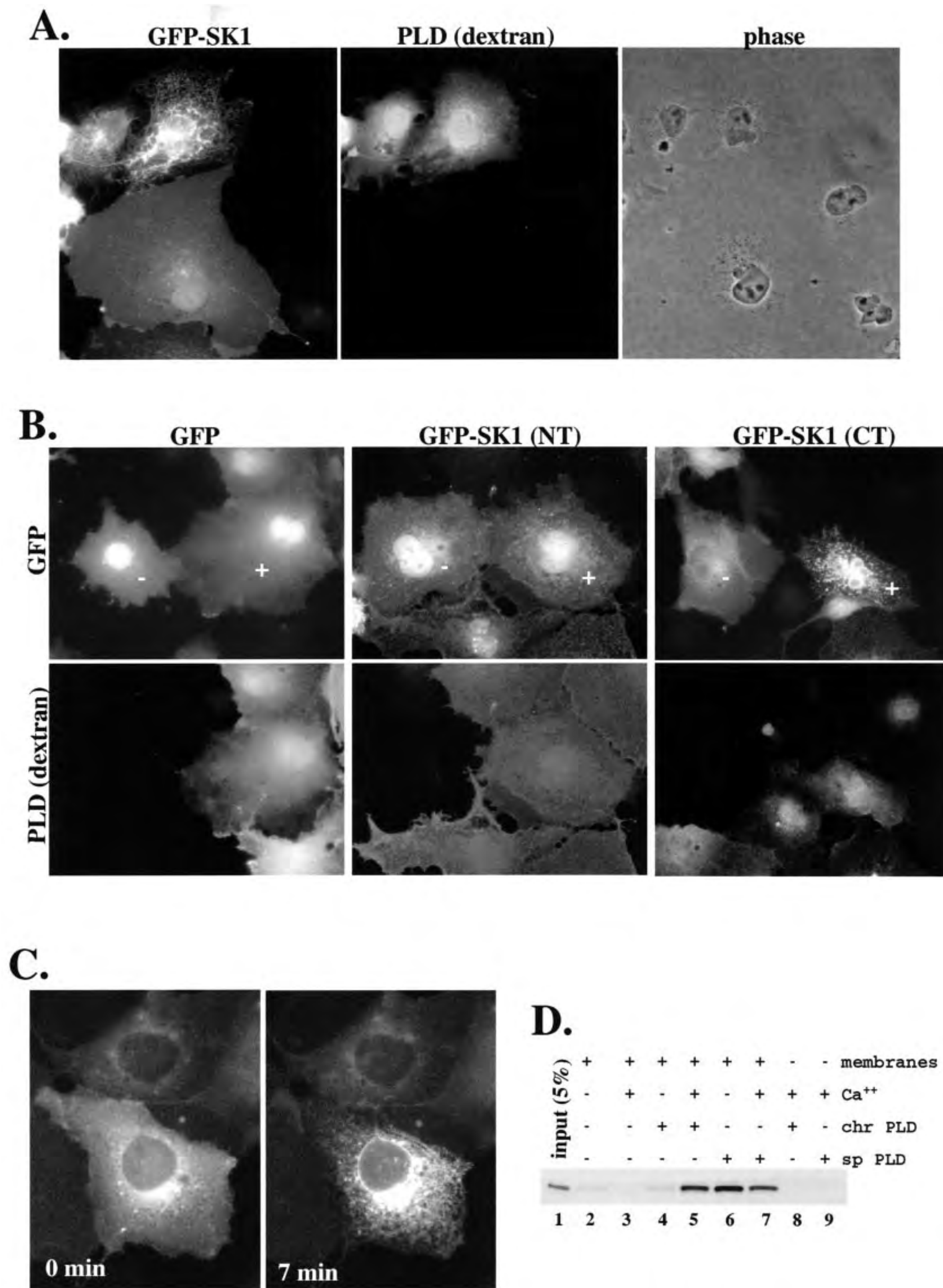


FIG. 7. **Membrane translocation of SK1 in cells microinjected with PLD.** *A*, COS cells expressing GFP-tagged SK1 were microinjected with a solution of bacterial PLD (*Streptomyces* sp.) containing biotinylated dextran. After recovery at 37 °C for 15 min, the cells were fixed and stained with avidin-Cy3 to mark the microinjected cells. Notice that PLD microinjection induces translocation of SK1 from the cytosol to perinuclear membranes. *B*, similar experiment as in *A*, but cells were expressing either GFP alone or GFP-tagged SK1 fragments as shown in Fig. 5. Notice that only the GFP-tagged C-terminal fragment of SK1 responds to PLD microinjection. *C*, PLD-induced SK1 translocation is time-dependent. The microinjected cell was observed by live imaging for 15 min. Two images immediately after microinjection and 7 min later are shown. *D*, translocation of SK1 to PLD-treated membranes showing that, unlike the *S. chromofuscus* PLD, the *Streptomyces* sp. PLD does not require calcium to induce translocation. This enzyme was used in all microinjections. Conditions for this binding experiment are as shown in Fig. 3*A*.

in vitro work to show that this is the case. From experiments performed *in vitro* we show that SK1 translocates to isolated cellular membranes or to liposomes of defined composition upon PA formation, and it binds to pure PA matrices both in complex lysate mixtures and in purified recombinant form. From experiments using intact cells we show that GFP-SK1 translocates very rapidly from the cytosol to perinuclear membranes upon microinjection of pure bacterial PLD. In addition, GFP-SK1 becomes partially membrane-localized in RBL-2H3 mast cells upon receptor stimulation that elevates endogenous PLD activity and PA levels. Although we do not yet know the exact identity of the PA binding determinant on SK1, our

Downloaded from http://www.jbc.org/ at Babraham Institute on July 17, 2015

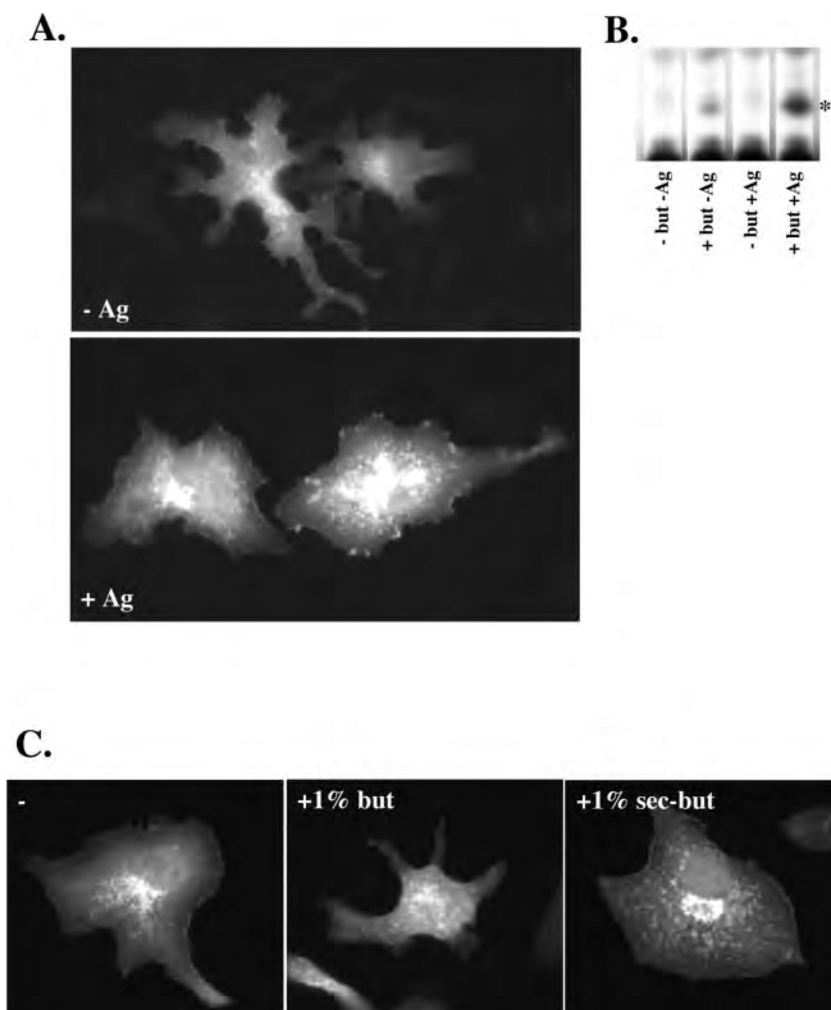


FIG. 8. **Membrane translocation of SK1 in cells following receptor activation of endogenous PLD.** *A*, RBL-2H3 cells were transfected with GFP-tagged SK1. The FcεRI was sensitized by incubation for 16 h with 25 ng/ml anti-DNP IgE. The following day, receptor was cross-linked with 25 μg/ml DNP-bovine serum albumin in some samples (+*Ag*) or left untreated (−*Ag*) for 25 min at 37 °C. At the end of treatments, cells were fixed and examined for fluorescence. *B*, RBL-2H3 cells were labeled overnight with [^{3}H]palmitate in the presence of 25 ng/ml anti-DNP IgE. The following morning, labeling medium was replaced with fresh solution that contained 1% butanol (+*but*) and/or 25 μg/ml DNP-bovine serum albumin (+*Ag*) as indicated, for 30 min at 37 °C. At the end of 30 min, lipids were extracted and analyzed by thin layer chromatography shown here. The position of phosphatidylbutanol (a signature PLD product) is shown with an *asterisk*. *C*, RBL-2H3 cells were treated as in *A*, but before antigen stimulation butanol or *sec*-butanol at 1% was included in the medium as indicated. Note that the primary alcohol blocks SK1 perinuclear and plasma membrane translocation.

experiments suggest that it is contained within the C-terminal half of the protein, away from the diacylglycerol kinase-like domain that is found within the catalytic region.

The membrane sites of SK1 translocation upon elevation of PA are fairly heterogeneous and depend to some degree on the method of PA elevation. When PA is elevated via activation of endogenous PLD, most SK1 translocates to a perinuclear region (which co-localizes with endosomal markers and partially overlaps with the Golgi) as well as to the plasma membrane. This perinuclear and plasma membrane distribution is consistent with previous reports on SK1 localization (18, 32). When PA levels are elevated by PLD microinjection, we observed, in addition to perinuclear sites, SK1 translocation to the ER. The significance of this is unknown and may be an artifact of the preferred site of action of the bacterial enzyme. We note, however, that the yeast equivalent of SK1 is an ER enzyme (33) that the hydrolases, which degrade S1P, are also ER enzymes (34, 35) and that S1P was able to mobilize calcium from isolated microsomal membranes, which are derived from the ER (36) or from thapsigargin-sensitive calcium ER stores (37). In addition, immunofluorescence staining of endogenous SK1 under basal conditions appears to partially localize the protein in an ER-like compartment (18). Therefore, it is possible that both the perinuclear/plasma membrane sites and the ER are *bona fide* loci of SK1 translocation under some conditions.

Our recent focus on potential PA-binding proteins has allowed us to examine simultaneously a large number of potential targets, and we are beginning to discern some differences in PA binding among the different proteins. For example, most putative PA targets bind immobilized PA to a similar extent. In a typical experiment, and for about 5 nmol of PA used, ~5% of the input protein is found in the PA-bound fraction. The fraction of SK1 that is bound to PA is 5- to 8-fold higher and constitutes the highest we have seen among the potential PA targets. In addition, when we labeled total cytosol with [^{35}S]methionine and then carried out a membrane translocation experiment such as shown in Fig. 3, none of the translocated proteins showed as strong membrane enrichment as SK1 (data not shown). Based on these results, we suggest that the affinity of SK1 for PA maybe one of the strongest among cellular PA targets.

The Significance of the SK1 PA Interaction—S1P is elevated in cells as a consequence of stimulation by a variety of growth factors, bioactive lipids, and G protein-coupled receptor agonists (reviewed in Refs. 38 and 39). Once formed, S1P can either be secreted and activate cell surface receptors in an autocrine/paracrine fashion or it can activate signaling pathways intracellularly without being exported (reviewed in Ref. 32). Both modes of S1P action result in distinct and important physiological consequences. Binding of secreted S1P to its receptors can affect proliferation, migration, and differentiation, whereas intracellular S1P regulates calcium homeostasis and antagonizes apoptotic signals (32, 40). These properties of S1P make the regulation of its formation of considerable interest. Our finding that SK1 is a PA effector provides a potential mechanism for the stimulation of SK1 by extracellular stimuli. We propose that, in some cases, stimulation of PLD activity by extracellular signals leads to elevated intracellular PA levels

Downloaded from http://www.jbc.org/ at Babraham Institute on July 17, 2015

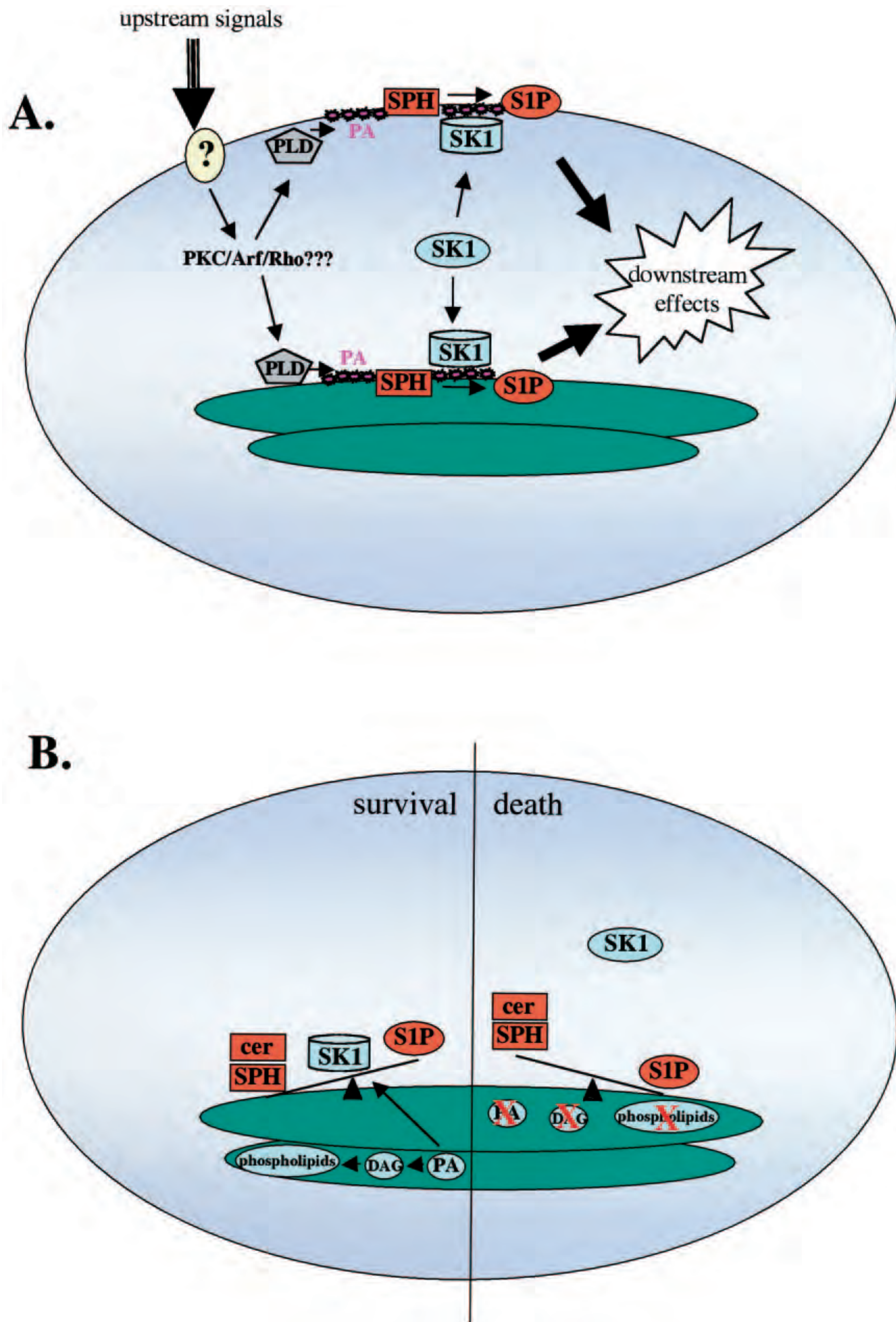


FIG. 9. **The SK1-PA interaction in the context of two signaling pathways.** *A*, for pathways where extracellular signals lead to elevated levels of intracellular S1P, we propose that PLD activation is a potentially critical intermediate. In this model, extracellular signals activate endogenous PLD (perhaps via a combination of the three known PLD protein activators, PKC, Arf, or Rho) leading to elevated PA levels at defined sites either on the plasma membrane or on internal organelles. SK1 translocates from the cytosol to the PA-enriched membranes where it encounters its substrate and forms S1P, leading to S1P-mediated downstream effects. *B*, in non-PLD-dependent pathways, the SK1-PA interaction may constitute a way to reinforce survival signals as predicted by the sphingolipid rheostat hypothesis. In normally growing cells, phospholipid biosynthesis would generate a constant flux of PA as an intermediate. This PA could also be used to attract SK1 to membranes for the generation of S1P, a survival signal (*left panel*). Under conditions of cellular stress, cessation in phospholipid biosynthesis would deplete membranes of PA. This may lead to cytosolic re-distribution of SK1, a reduction of S1P formation and an increase in the levels of SPH and ceramide, two lipids known to constitute apoptotic signals (*right panel*).

Downloaded from http://www.jbc.org/ at Babraham Institute on July 17, 2015

(either at the plasma membrane or internal compartments), which would in turn lead to PA-mediated translocation of cytosolic SK1 to membranes enriched in its substrate, sphingosine (Fig. 9*A*). In this view, elevation of S1P levels by extracellular stimuli would be a direct consequence of PA-induced proximity of the enzyme to its substrate. For example, following receptor cross-linking in immune cells, it has been reported that PLD activation precedes and correlates well with SK1 activity (reviewed in Ref. 17). One way to explain this result based on our observations is to hypothesize that receptor cross-linking leads to elevated PLD activity, which in turn induces membrane translocation of SK1. Although this general mechanism for translocation is lipid-based and need not involve additional components, it is of course entirely likely that additional factors may enhance or confer specificity to the SK1 translocation. For example, calcium/calmodulin (41), PKC (18), ERK 1/2 (42), TRAF2 (43), RPK118 (44), and a novel protein related to protein kinase A-anchoring protein (45) have all been shown to affect SK1 translocation and activity either via direct protein-protein interactions or via phosphorylation.

The SK1-PA interaction may be relevant in both PLD-dependent and independent settings, especially in the regulation of growth and survival. The cellular balance between S1P and SPH/ceramide has been proposed to constitute a "sphingolipid rheostat" that regulates cell survival/death decisions with S1P on the side of survival (46), and it is interesting that both PLD and SK1 are implicated in cell survival (47, 40). It is possible therefore that PLD activation in healthy cells results in increased synthesis of S1P, thus reinforcing a survival decision. Conversely, reduced PLD activity in dying cells would prevent SK1 translocation to membranes, resulting in a drop in S1P, a concomitant increase in the levels of apoptotic SPH/ceramide, and a reinforcement of the death decision. However, the possibility that SK1 translocates to PA-enriched membranes in settings not involving PLD activation must also be considered. PA is a major intermediate in phospholipid biosynthesis (48), and its cellular levels may be used as an indicator of the physiological state of a cell. In support of this, there is a strong correlation between inhibition of phosphatidylcholine synthesis and apoptosis in a variety of experimental systems (49–52). It is possible that PA formation as part of normal lipid biosynthesis would constitute a survival signal that enables membrane translocation of SK1 and increased synthesis of S1P (Fig. 9*B*). Conversely, cessation in phospholipid biosynthesis as part of a death decision would deplete membranes of PA leading to SK1 redistribution to the cytosol and a drop in the levels of S1P. In this model, the SK1-PA interaction would allow a functional connection between the sphingolipid rheostat and the phospholipid steady state.

Acknowledgments—We thank our colleagues at Babraham (Len Stephens and Phill Hawkins) for support and the chemistry team of Prof Andrew Holmes at the University of Cambridge for making the lipid-coupled beads. We also thank Mike Frohman for a gift of the tet-inducible PLD1 cells.

REFERENCES

1. Ktistakis, N., Delon, C., Manifava, M., Wood, E., Ganley, I., and Sugars, J. (2003) *Biochem. Soc. Trans.* **31,** 94–97
2. Exton, J. (1999) *Biochim. Biophys. Acta* **1439,** 121–133
3. Ghosh, S., Strum, J. C., Sciorra, V. A., Daniel, L., and Bell, R. M. (1996) *J. Biol. Chem.* **271,** 8472–8480
4. Rizzo, M. A., Shome, K., Watkins, S. C., and Romero, G. (2000) *J. Biol. Chem.* **275,** 23911–23918
5. Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A., and Chen, J. (2001) *Science* **294,** 1942–1945
6. Frank, C., Keilhack, H., Opitz, F., Zschoring, O., and Bohmer, F. (1999) *Biochemistry* **38,** 11993–12002
7. Jones, J., and Hannun, Y. (2002) *J. Biol. Chem.* **277,** 15530–15538
8. Moritz, A., De Graan, P. N., Gispen, W. H., and Wirtz, K. W. (1992) *J. Biol. Chem.* **267,** 7207–7210
9. Jenkins, G., Fisette, P., and Anderson, R. (1994) *J. Biol. Chem.* **269,** 11547–11554
10. Litosch, I. (2000) *Biochemistry* **39,** 7736–7743
11. Grange, M., Sette, C., Cuomo, M., Conti, M., Lagarde, M., Prigent, A., and Nemoz, G. (2000) *J. Biol. Chem.* **275,** 33379–33387
12. Baillie, G., Huston, E., Scotland, G., Hodgkin, M., Gall, I., Peden, A., MacKenzie, C., Houslay, E., Currie, R., Pettitt, T., Walmsey, A., Wakelam, M., Warwicker, J., and Houslay, M. (2002) *J. Biol. Chem.* **277,** 28298–28309
13. Jose Lopez-Andreo, M., Gomez-Fernandez, J., and Corbalan-Garcia, S. (2003) *Mol. Biol. Cell* **14,** 4885–4895
14. Karathanassis, D., Stahelin, R., Bravo, J., Perisic, O., Pacold, C., Cho, W., and Williams, R. (2002) *EMBO J.* **21,** 5057–5068
15. Spiegel, S., and Milstien, S. (2002) *J. Biol. Chem.* **277,** 25851–25854
16. Olivera, A., Rosenthal, J., and Spiegel, S. (1996) *J. Cell. Biochem.* **60,** 529–537
17. Melendez, A., and Allen, J. (2002) *Semin Immunol.* **14,** 49–55
18. Johnson, K., Becker, K., Facchinetti, M., Hannun, Y., and Obeid, L. (2002) *J. Biol. Chem.* **277,** 35257–35262
19. Manifava, M., Sugars, J., and Ktistakis, N. T. (1999) *J. Biol. Chem.* **274,** 1072–1077
20. Ktistakis, N. T., Brown, H. A., Sternweis, P. C., and Roth, M. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 4952–4956
21. Ktistakis, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C., and Roth, M. G. (1996) *J. Cell Biol.* **134,** 295–306
22. Manifava, M., Thuring, J. W. J. F., Lim, Z. Y., Packman, L., Holmes, A. B., and Ktistakis, N. T. (2001) *J. Biol. Chem.* **276,** 8987–8994
23. Lim, Z., Thuring, J., Holmes, A., Manifava, M., and Ktistakis, N. (2002) *J. Chem. Soc. Perkin Trans. 1* **2002,** 1067–1075
24. Bi, K., Roth, M. G., and Ktistakis, N. T. (1997) *Curr. Biol.* **7,** 301–307
25. Yamamoto, I., Konto, A., Handa, T., and Miyajima, K. (1995) *Biochim. Biophys. Acta* **1233,** 21–26
26. Krugmann, S., Anderson, K., Ridley, S., Risso, N., McGregor, A., Coadwell, J., Davidson, K., Equinoa, A., Ellson, C., Lipp, P., Manifava, M., Ktistakis, N., Painter, G., Thuring, J., Cooper, M., Lim, Z., Holmes, A., Dove, S., Michell, R., Grewal, A., Nazarian, A., Erdjument-Bromage, H., Tempst, P., Stephens, L., and Hawkins, P. (2002) *Mol. Cell* **9,** 95–108
27. Pitson, S., Moretti, P., Zebol, J., Xia, P., Gamble, J., Vadas, M., D'Andrea, R., and Wattenberg, B. (2000) *J. Biol. Chem.* **275,** 33945–33950
28. Pitson, S., Moretti, P., Zebol, J., Zareie, R., Derian, C., Darrow, A., Qi, J., D'Andrea, R., Bagley, C., Vadas, M., and Wattenberg, B. (2002) *J. Biol. Chem.* **277,** 49545–49553
29. Dinh, T., and Kennerly, D. (1991) *Cell Regul.* **2,** 299–309
30. Lin, P., and Gilfillan, A. (1992) *Eur. J. Biochem.* **207,** 163–168
31. O'Luanaigh, N., Pardo, R., Fensome, A., Allen-Baume, V., Jones, D., Holt, M., and Cockcroft, S. (2002) *Mol. Biol. Cell* **13,** 3730–3746
32. Spiegel, S., and Milstien, S. (2003) *Nat. Rev. Mol. Cell. Biol.* **4,** 397–407
33. Funato, K., Lombardi, R., Vallee, B., and Riezman, H. (2003) *J. Biol. Chem.* **278,** 7325–7334
34. Le Stunff, H., Galve-Roperh, I., Peterson, C., Milstien, S., and Spiegel, S. (2002) *J. Cell Biol.* **158,** 1039–1049
35. Ogawa, C., Kihara, A., Gokoh, M., and Igarashi, Y. (2003) *J. Biol. Chem.* **278,** 1268–1272
36. Mattie, M., Brooker, G., and Spiegel, S. (1994) *J. Biol. Chem.* **269,** 3181–3188
37. Meyer zu Heringdorf, D., Liliom, K., Schaefer, M., Danneberg, K., Jaggar, J., Tigyi, G., and Jakobs, K. (2003) *FEBS Lett.* **554,** 443–449
38. Pyne, S., and Pyne, N. (2000) *Biochem. J.* **349,** 385–402
39. Olivera, A., and Spiegel, S. (2001) *Prostaglandins Other Lipid Mediat.* **64,** 123–134
40. Pyne, S., and Pyne, N. (2002) *Biochim. Biophys. Acta* **1582,** 121–131
41. Young, K., Willets, J., Parkinson, M., Bartlett, P., Spiegel, S., Nahorski, S., and Challiss, R. (2003) *Cell Calcium* **33,** 119–128
42. Pitson, S., Moretti, P., Zebol, J., Lynn, H., Xia, P., Vadas, M., and Wattenberg, B. (2003) *EMBO J.* **22,** 5491–5500
43. Xia, P., Wang, L., Moretti, P., Albanese, N., Chai, F., Pitson, S., D'Andrea, R., Gamble, J., and Vadas, M. (2002) *J. Biol. Chem.* **277,** 7996–8003
44. Hayashi, S., Okada, T., Igarashi, N., Fujita, T., Jahangeer, S., and Nakamura, S. (2002) *J. Biol. Chem.* **277,** 33319–33324
45. Lacana, E., Maceyka, M., Milstien, S., and Spiegel, S. (2002) *J. Biol. Chem.* **277,** 32947–32953
46. Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P., Coso, O., Gutkind, S., and Spiegel, S. (1996) *Nature* **381,** 800–803
47. Nozawa, Y. (2002) *Biochim. Biophys. Acta* **1585,** 77–86
48. Athenstaedt, K., and Daum, G. (1999) *Eur. J. Biochem.* **266,** 1–16
49. Anthony, M., Zhao, M., and Brindle, K. (1999) *J. Biol. Chem.* **274,** 19686–19692
50. Miguel, K., Pradines, A., Terce, F., Selmi, S., and Favre, G. (1998) *J. Biol. Chem.* **273,** 26179–26186
51. Ramos, R., Mouedden, M., Claro, E., and Jackowski, S. (2002) *Mol. Pharmacol.* **62,** 1068–1075
52. Yen, C., Mar, M., and Zeisel, S. (1999) *FASEB J.* **13,** 135–142

Downloaded from http://www.jbc.org/ at Babraham Institute on July 17, 2015

The Journal of Biological Chemistry
AFFINITY SITES
ASBMB
American Society for Biochemistry and Molecular Biology

Mechanisms of Signal Transduction:
Sphingosine Kinase 1 Is an Intracellular Effector of Phosphatidic Acid

Christine Delon, Maria Manifava, Eleanor Wood, Dawn Thompson, Sonja Krugmann, Susan Pyne and Nicholas T. Ktistakis
J. Biol. Chem. 2004, 279:44763-44774.
doi: 10.1074/jbc.M405771200 originally published online August 13, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405771200

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 31 of which can be accessed free at
http://www.jbc.org/content/279/43/44763.full.html#ref-list-1

Downloaded from http://www.jbc.org/ at Babraham Institute on July 17, 2015