Role of Phosphatidic Acid in the Coupling of the ERK Cascade

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The production of phosphatidic acid plays a crucial role in the activation of the ERK cascade. This role was linked to the binding of phosphatidate to a specific polybasic site within the kinase domain of Raf-1. Here we show that phosphatidate promotes ERK phosphorylation in intact cells but does not activate Raf in vitro. The kinase suppressor of Ras (KSR) contains a sequence homologous to the phosphatidate binding site of Raf-1. Direct binding of phosphatidate to synthetic peptides derived from the sequences of the binding domains of Raf-1 and KSR was demonstrated by spectroscopic techniques. The specificity of these interactions was confirmed using synthetic lipids and mutated peptides in which the core of the phosphatidic acid binding domain was disrupted. Insulin and exogenous dioleoyl phosphatidate induced a rapid translocation of a mouse KSR1-EGFP construct to the plasma membrane of HIRcB cells. Mutation of two arginines located in the core of the putative phosphatidate binding site abolished dioleoyl phosphatidate- and insulin-induced translocation of KSR1. Overexpression of the mutant KSR1 in HIRcB cells inhibited insulin-dependent MEK and ERK phosphorylation. The addition of dioleoyl phosphatidate or insulin increased the co-localization of KSR1 and H-Ras and promoted the formation of plasma membrane patches enriched in both proteins and phosphatidic acid. These results, in conjunction with our previous work, suggest the formation of phosphatidate-enriched membrane microdomains that contain all components of the ERK cascade. We propose that these domains act as molecular scaffolds in the coupling of signaling events.

Although the idea that phosphatidic acid (PA) is an important lipid second messenger seems to be widely accepted, the actual functions of this lipid in signal transduction are still poorly understood. Several physiological roles for PA have been proposed in the past. These include regulation of protein and lipid phosphorylation (1–3), regulation of cAMP degradation (4), activation of oxidative processes (3, 5), and modulation of membrane traffic (6–9). Many of these functions are mediated by the direct interaction of PA with specific target proteins. Thus, PA appears to function in a manner analogous to other lipid second messengers (i.e. by promoting the binding of target proteins to specific regions of the cell membrane).

Direct binding of PA has been demonstrated in a small subset of proteins. The interactions of PA with Raf-1 have been mapped to a 35-amino acid stretch within the kinase domain (10–13), whereas the binding of PA to mTOR involves Arg2109, a residue located in the vicinity of the rapamycin binding domain (14, 15). Putative binding sites for the cyclic nucleotide phosphodiesterase PDE4D3 (4) and the protein-tyrosine phosphatase SHP-1 (16) have also been described. In general, these PA binding domains bear little sequence similarity to each other, except for the fact that they all contain at least one polybasic motif.

Previous work from our laboratory has demonstrated that PA binding is essential for the recruitment of Raf-1 to the plasma membrane and its subsequent activation (12, 13). This conclusion was based on the findings that treatment with exogenous PA induced a rapid, transient translocation of Raf-1 to the plasma membrane and that mutation of key basic residues located in the putative PA binding region (PABR) of Raf-1 prevented Raf-1 translocation and activation. We also showed that interference with PA production or expression of a PA scavenger peptide based on the Raf-1 PABR resulted in the inhibition of insulin-dependent ERK phosphorylation (13).

More recent work has demonstrated a second PA target in the Ras/Raf/ERK cascade: the Ras guanine nucleotide exchange factor Sos, which binds PA via its pleckstrin homology domain (17). Thus, PA production appears to be an absolute requirement for the coupling of the ERK cascade in cell membranes. However, neither MEK1/2 nor ERK1/2 possess well defined membrane binding domains. Thus, the question of how the remaining components of the cascade assemble on lipid membranes remains an open question.

The scaffolding proteins KSR1 and KSR2 (kinase suppressor of Ras 1 and 2) bear substantial structural similarities with the Raf family of protein kinases. KSR1 binds MEK1, ERK1/2, and perhaps Raf-1 (18–20). The C-terminal domain of KSR1 is remarkably similar to the kinase domain of Raf-1 (21, 22). KSR1 has been shown to bind lipids via an N-terminal cysteine-rich domain (23). Moreover, KSR1 contains a sequence homologous...
to the PABR of Raf-1. Thus, we hypothesized that PA modulates the scaffolding function of KSR proteins by promoting their association to PA-enriched regions that contain the remaining elements of the ERK cascade.

In this paper, we compare the interactions of PA with the PABR of Raf-1 and KSR1. Our data demonstrate direct, specific binding of PA to the PABR of both proteins. Furthermore, we provide substantial evidence in support of the hypothesis that PA modulates the binding of KSR1 to membranes and that this binding is essential for the scaffolding role of KSR in the coupling of the ERK kinase cascade.

**MATERIALS AND METHODS**

**Cells, cDNAs, and Lipids Used**—HIREc cells were cultured in Dulbecco’s modified Eagle’s medium/F-12, 10% fetal calf serum containing 100 μM methotrexate. NIH-3T3 and HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum. Plasmids encoding mKSR1 were kindly supplied by Dr. R. Kolesnick and Dr. K-L. Guan. KSR1 was subcloned into pEGFP-C1 using the EcoRI and SalI restriction sites. HA-KSR1 was generated by subcloning into pCMV-HA between the NotI and BglII sites. R612A/R615A KSR1 (DM-KSR1) was made by the four-primer method. H-Ras was subcloned in dsRed2 as described (24). All sequences were confirmed. Cells were transfected using Lipofectamine 2000 or Fugene 6 as per the instructions supplied by the manufacturers. All lipids were obtained from Avanti as chloroform solutions. Lipids were aliquoted appropriately, dried, and resuspended in Tris-buffered saline.

**Peptides**—All peptides were synthesized at the Molecular Medicine Institute Peptide Synthesis Facility of the University of Pittsburgh. All peptides were purified by high pressure liquid chromatography, and all sequences were verified by mass spectrometry analysis. The purity of the preparations used in this work was greater than 95%. The sequences of the peptides used are shown in Fig. 3A.

**Fluorescence Spectroscopy**—Tryptophan fluorescence data were acquired using a Fluoromax-3 spectrofluorometer equipped with single grating excitation and emission monochromators. The peptides were dissolved at concentrations between 0.6 and 6 μM in 50 mM Tris (pH 7.5) containing 100 mM NaCl and 1 mM EGTA. Concentrated lipid stocks (5 mM) were prepared by dissolving dried lipid films in 18 mM CHAPS. These preparations were clear micelles. Lipid vesicles were prepared by dilution of the stock lipid solutions by at least 100-fold (final [CHAPS] < 200 μM), followed by sonication using a microtip sonicator. The critical micelle concentration of CHAPS is 6.2 mM in 100 mM NaCl (25). Thus, the concentration of CHAPS in the diluted lipid samples was at least 50-fold lower than the critical micelle concentration of the detergent. Under these conditions, the solubilized lipids aggregate to form vesicular structures (26). Samples were excited at 280 nm, and emission spectra were measured in the 300–400 nm range. Corrections were made using control solutions containing lipid only or peptide only as blanks. All titration data were collected at a single wavelength (325 nm). Each point represents a 5-s average. Titration data were analyzed using GraphPad Prism.

**Circular Dichroism**—CD measurements were taken using peptide solutions ranging in concentration from 0.2 to 0.5 mg/mL, in 50 mM Tris, pH 7.4, at 25°C, in the presence or absence of 50 μM lipid and 18 mM CHAPS. All samples were sonicated for 5 min just prior to analysis. Measurements were taken using an AVIV instrument built on a Varian Cary 60 platform using AVIV data acquisition and data processing software. The data reported reflect an average of at least 10 spectra/sample. CD data were exported to Excel and analyzed using CDSTR, from DICHROWEB (available on the World Wide Web) using the built in data bases available on the server (reference set 4 for the range 190–240 nm; data truncated at 240 nm). These analyses were confirmed using CDFIT (available on the World Wide Web).

**Fluorescence Microscopy**—Confocal microscopy data were obtained with a Zeiss LSM5 confocal microscope. Total internal reflection fluorescence (TIRF) microscopy data were obtained using a home-designed instrument based on an Olympus TIRF platform. This instrument was equipped with a Hamamatsu Orca ER camera, two solid state lasers (491 and 561 nm), and an acoustic-optical tunable filter. The laser launch/acoustic-optical tunable filter module was manufactured by VisiTech International. The data acquisition system was controlled by SimplePCI (Hamamatsu, Inc). Most experiments were performed at room temperature.

The cells used in these experiments were grown on coverslips or using 35 mm MatTek glass bottom dishes. The cells were transfected with fluorescent constructs 24–48 h prior to the experiment. All cells were serum-starved overnight. Fluorescence data were exported to ImageJ for processing and analysis. Co-localization data were analyzed using an image correlation plug-in for ImageJ.

**Detection of Phosphatidic Acid**—Phosphatidic acid was detected using a biotinylated derivative of Raf-1-PABR. This derivative was used at a final concentration of 2 μg/mL. To detect PA, the cells were incubated with serum-free medium, 100 μM dioleoyl PA (DOPA), or 200 nM insulin for 2–5 min. The incubation was stopped by rapid chilling, followed by a rapid wash with Tris-buffered saline. The cells were then fixed with 3% buffered formaldehyde for 5 min at room temperature, blocked with 3% bovine albumin (1 h), and incubated with the biotinylated-PABR reagent for 4 h. Unbound biotinylated PABR was removed by three 5-min washes with phosphate-buffered saline. The bound peptide was detected with Cy5-conjugated streptavidin. Most experiments were performed with HIREc cells pretransfected with KSR1-EGFP and dsRed2-H-Ras in order to investigate co-localization of all three components.

Co-localization experiments were done using a Zeiss LSM5 confocal microscope. Detection of KSR1-EGFP and dsRed2-Ras was done, measuring the intrinsic fluorescence of the transfected proteins. Biotinylated PABR was detected using Cy5-tagged streptavidin. All confocal scans were done in the Sequential mode of the instrument in order to avoid bleed-through artifacts. Potential bleed through was examined using single-label preparations and found to be negligible under the conditions tested.

**ERK Phosphorylation**—Live cell ERK phosphorylation experiments were performed using cells that had been serum-starved overnight. The DOPA suspensions used to treat intact cells...
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FIGURE 1. Phosphatidic acid induces ERK phosphorylation in various cell lines. Cell cultures were serum-starved for 24 h and treated as specified in the figure in the presence of 10 mg/ml bovine serum albumin. DOPA liposomes were sonicated immediately prior to their use. ERK and MEK phosphorylation were determined as described under “Materials and Methods.” A, DOPA induces ERK phosphorylation in HIRcB, HEK-293, and NIH-3T3 cells. B, time course of the activation of ERK and MEK by DOPA in NIH-3T3 cells. C, in vitro Raf activity assay. Recombinant v-Raf was incubated with recombinant MEK1 and ATP using a commercial kit. The assay was performed as per the manufacturer’s instructions with or without the addition of DOPA or CHAPS at the concentrations indicated in the figure.

FIGURE 2. Comparison of the sequences of Raf and KSR. The polybasic motifs are underlined. Human (h) and mouse (m) sequences are shown.

were prepared from stock 1 mM suspensions prepared in phosphate-buffered saline containing 10 mg/ml bovine serum albumin. These suspensions were sonicated for 12 min using a horn sonicator just before their use. ERK phosphorylation was determined by Western blotting with commercial specific antibodies, as described before (12, 13).

RESULTS

The Addition of Exogenous PA Promotes ERK Phosphorylation—We have previously reported that PA does not stimulate the phosphorylation of ERK (12). Those studies were performed using aqueous suspensions of dipalmitoyl-PA, DOPA, or palmitoleoyl-oleoyl phosphatidic acid. In all cases, the lipid was prepared as an aqueous suspension and used without further treatment. However, PA tends to form multilamellar vesicles, large structures in which a very small fraction of the lipid is contained in the outer monolayer (27, 28). Therefore, we reexamined these results using sonicated DOPA vesicles. This procedure results in the formation of small unilamellar vesicles, in which roughly 50% of the lipid is exposed to the solvent (27, 28). In contrast with our previous results, the addition of unilamellar DOPA vesicles induced ERK phosphorylation responses in HIRcB, HEK-293, and NIH-3T3 cells (Fig. 1A). These effects were dose-dependent and strongly influenced by the cell type. As shown, in HIRcB cells, the effects at concentrations lower than 300 μM DOPA were small but detectable. In contrast, 100 μM DOPA had maximal effects in NIH-3T3 and HEK-293 cells. The effect of DOPA on ERK phosphorylation was rapid and sustained (Fig. 1B). Importantly, the addition of DOPA did not increase the in vitro activity of Raf assayed with recombinant v-Raf and MEK1 (Fig. 1C). Thus, the effects of PA are not a consequence of a direct activation of Raf. However, because of the high concentrations of DOPA used in these studies, we cannot rule out potential indirect actions of exogenous PA. PA can be converted to diacylglycerol or lyso-PA by the actions of PA-phospholipases (29) and phospholipase A (30, 31), respectively. Both diacylglycerol and lyso-PA may activate the ERK cascade by independent signaling mechanisms, and these effects have not been ruled out in our studies.

The PABRs of Raf and KSR1 Bind Phosphatidic Acid—Independently of the potential indirect effects of PA, previous work from our laboratory demonstrated that PA production is necessary for the recruitment of Raf-1 to the membrane and the subsequent activation of the ERK cascade (12, 13). Two additional potential targets of PA are the scaffolding proteins KSR1 and KSR2, which also play a major role in the coupling of the ERK cascade (18, 19). A comparison of the sequences of the putative PABRs of KSR1, KSR2, Raf-1, B-Raf, and A-Raf is shown in Fig. 2. As shown, KSR and Raf proteins share a very high degree of homology within this region. An important characteristic of these sequences is the presence of a polybasic motif (highlighted in Fig. 2). Mutation of both arginine residues within this core renders Raf-1 inactive in the coupling of the ERK kinase cascade (13).

The binding of proteins and peptides to PA-containing phospholipid vesicles has been studied using fractionation procedures (17), enzyme-linked immunosorbent assay-like techniques in which the lipid is bound to the solid matrix (10, 11), and plasmon resonance methods in which the lipid is immobilized to the solid matrix of lipophilic biosensor chips (32). The problem with these techniques is that the lipid is always used in very large excess, such that the method measures partition coefficients rather than actual dissociation equilibrium constants. This hinders the direct comparison of quantitative data obtained by the different procedures. We decided, instead, to study the direct binding of the PABRs of Raf-1 and KSR1 using modified peptides in which one phenylalanine residue downstream of the polybasic motif was replaced with a tryptophan (see Fig. 3A). Tryptophan is a fluorescent amino acid that exhibits exquisite sensitivity to changes in the polarity of its environment (33–36). Thus, the binding of PA to Raf-1-PABR
and KSR1-PABR can be measured directly following the fluorescence of the modified tryptophan using lipid and peptide concentrations that are of the order of magnitude of the PABR-PA $K_d$. This is clearly illustrated in Fig. 3B; the addition of DOPA blue-shifted the fluorescence of the KSR1-PABR peptide. In comparison, the double arginine-to-alanine mutant (DM-KSR1-PABR) was insensitive to the addition of DOPA (up to 10 mM; Fig. 3B). It should be noted that DM-KSR1-PABR was blue-shifted in comparison with the wild type form. This is possibly a consequence of the increased hydrophobicity of the peptide after replacement of the two arginines of the polybasic core.

These constructs were also used to determine the affinity and specificity of Raf-1-PABR and KSR1-PABR to various lipids. The binding curves of these peptides to DOPA are shown in Fig. 3C. As shown, Raf-1-PABR bound DOPA with a $K_d$ of 78 nM, a figure comparable with the affinity of the Ras binding domain of Raf-1 for H-Ras (160 nM) (37). The binding of the PABR to PA is driven primarily by the electrostatic interactions of a polybasic motif embedded in the sequence (RKTRH). This is supported by the complete lack of DOPA binding of double mutants in which both Arg residues were replaced by Ala (Fig. 3C). The affinity of KSR1-PABR for DOPA was much lower ($K_d = 1.2 \mu M$). To demonstrate that the difference in affinities is primarily a consequence of the different electrostatic charge of the polybasic motifs of both PABRs (Raf-1, RKTRH; KSR1, RQTRH), we examined the binding of a mutated KSR1 peptide in which the glutamine had been replaced by a lysine (KSR-K PABR). As expected, this modified
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Further analysis of the interactions of the PABR peptides with PA was done by circular dichroism. This spectroscopic technique is useful to determine the secondary structure of proteins and peptides. The ellipticity data shown in Fig. 4A were obtained using 0.5 mg/ml WT-Raf-1-PABR and lipid-detergent micelles containing 50 μM lipid in 18 mM CHAPS. Micellar lipid preparations were selected to minimize the interference of large lipid vesicles with the assay. In the absence of lipids, WT-Raf-1-PABR adopted a disorganized random coil structure. The addition of CHAPS or phosphatidylcholine-CHAPS micelles accentuated this unstructured conformation, as evidenced by the more pronounced minimum at 200 nm. However, the addition of DOPA-CHAPS micelles induced a dramatic structural change; the minimum at 200 nm disappeared, being replaced by a distinctive minimum at 220 nm, characteristic of a β-sheet secondary structure. Identical measurements were performed using the DM-Raf-1-PABR peptide. As shown in Fig. 4A, the mutant did not change conformation in the presence of DOPA. The behavior of the KSR1-PABR peptides was essentially identical to that of their Raf-1-derived counterparts (Fig. 4B). These results demonstrate convincingly that the PABR peptides interact directly with PA and that these interactions induce major conformational changes in the peptide.

The Interactions of Raf-1-PABR and KSR1-PABR with Lipids Are Selective—The sequences of the Raf-1 and KSR1 PABRs contain a polybasic motif flanked by two hydrophobic regions. These studies clearly demonstrate that the polybasic motifs play a central role in the binding of PA. The data shown above also suggest a certain degree of specificity. CHAPS and phosphatidylcholine-CHAPS micelles do not induce significant conformational changes in WT-Raf-1-PABR, as demonstrated by the very minor effects that these lipid structures have on the CD spectrum of the peptide. Furthermore, these data also demonstrate that interactions of WT-Raf-1-PABR with DOPA are not limited by simple electrostatic interactions between the polybasic motif and the negatively charged head group, since the addition of CHAPS, an anionic detergent, failed to induce conformational changes similar to those observed with DOPA.

To examine the specificity of the interactions of the PABR peptides with lipids, we determined the binding of several lipids using the tryptophan fluorescence method. The results are summarized in Table 1. $K_d$ values reported with only a lower limit (such as $K_d \gg 10 \mu M$) indicate experiments in which the lipids were used at the stated limit (i.e. 10 μM) either without

### Table 1

| Lipid                     | $K_d$  |
|---------------------------|--------|
| DOPA                      | 1266 ± 262 nM, 78 ± 12 nM |
| Palmitoleoyl-oleoyl phosphatidic acid | 841 ± 144 nM, 54 ± 18 nM |
| Dipalmityl-oleoyl phosphatidic acid | >10 μM, 2210 ± 2210 nM |
| Dihexanoyl phosphatidic acid | 1242 ± 131 nM, 102 ± 22 nM |
| Dioleoyl phosphatidylserine | >10 μM, >10 μM |
| Egg phosphatidylcholine    | >10 μM, >10 μM |
| Phosphatidylinositol       | >1 μM, >1 μM |
| Phosphatidylinositol 3,4-bisphosphate | >1 μM, >1 μM |
| Phosphatidylinositol 4,5-bisphosphate | >1 μM, >1 μM |
| Phosphatidylinositol 3,4,5-trisphosphate | >1 μM, >1 μM |

PABR bound DOPA with greater affinity. However, the affinity of the Raf-1-derived peptide was still higher, suggesting that other components may contribute to the affinity of the Raf-1 PABR for PA.
evidence of binding or very far from saturation, thus not allowing for a reliable calculation of the binding affinity. In the cases of egg phosphatidylcholine and dioleoyl phosphatidylserine, lipid concentrations greater than 3 μM were impractical because the light scattering of the sample interfered significantly with the fluorescence measurements. The phosphatidylinositol derivatives were used at a maximal concentration of 1 μM. WT-Raf-1-PABR exhibited much higher affinity for all forms of PA than WT-KSR1-PABR. Both PABR peptides showed significant selectivity for PA molecules containing long chain fatty acids. The much lower affinities observed for phosphatidylinositol derivatives and phosphatidylserine demonstrate that the interactions of the PABR peptides with PA are specific and selective.

PA Binding Is Required for the Scaffolding Role of KSR1—To determine the role of PA binding in the biological role of KSR1, a double mutation was introduced, replacing Arg<sup>612</sup> and Arg<sup>615</sup> by alanines. This mutant KSR1 (DM-KSR1) was transfected to HIRcB cells. Wild-type KSR1 and empty vector were used as controls.

The results shown in Fig. 5A demonstrate that DM-KSR1 expression significantly impairs the ability of insulin to stimulate MEK and ERK phosphorylation in HIRcB cells. To demonstrate that DM-KSR1 is acting as a dominant negative mutant, HIRcB cells were transfected with either 0.5 or 5 μg of plasmid coding for the mutant protein. The results of these treatments on insulin-induced ERK phosphorylation demonstrate that the effect of the mutant KSR1 is dose-dependent (Fig. 5C).

The Addition of Exogenous PA Induces the Translocation of KSR1 to the Plasma Membrane—We examined whether the recruitment of KSR1 to the plasma membrane was regulated by the presence of PA. These experiments were done using WT- and DM-KSR1 fused with EGFP at the N terminus and various optical techniques. Confocal microscopy of cells transfected with EGFP-WT-KSR1 and EGFP-DM-KSR1 showed similar distributions; both constructs were uniformly distributed throughout the cytosol, with small patches of greater intensity near the cell surface (see Fig. S1, A and B). TIRF studies on the distribution of the fluorescent constructs demonstrated that WT-KSR1 localizes to patches on the plasma membrane of naive HIRcB cells (Fig. 6A). The appearance of these patches was reminiscent of the distribution of cortical actin; however, the nature of the patches was not further analyzed.

The addition of 100 μM DOPA to the extracellular medium caused a minor but detectable translocation of the EGFP-WT-KSR1 construct (Fig. 6B). Importantly, DOPA-induced KSR1 recruitment was not uniform throughout the surface of the cell. This is shown in Fig. 6A, where the translocation of EGFP-WT-KSR1 in different regions of the cells was determined from TIRF data. It is clear from the data that the effects on the region of interest (ROI) labeled as ROI<sub>3</sub> (see position of the arrow in the right panel of Fig. 6A) is much more “active” in the recruitment of EGFP-WT-KSR1. A close-up of the process is shown in Fig. 6B. The data show that EGFP-WT-KSR1 transiently binds the plasma membrane after the addition of DOPA, forming...
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FIGURE 6. PA and insulin promote the translocation of KSR1 to the plasma membrane. HIRcB cells plated in 35-mm MatTek dishes were transfected with 2 μg of KSR1-EGFP using Fugene 6 and examined 48 h after transfection. Cells were serum-starved overnight before the imaging experiments. The image sequences shown in the figure are representative of at least five separate experiments. A, TIRF image of a fibroblast expressing WT-KSR1-EGFP. The arrows point to the ROI selected for the quantitative analysis shown in the left panel. As shown, the kinetics of the translocation of WT-KSR1-EGFP to the plasma membrane was not uniform throughout the cell surface. B, a close-up of the translocation of WT-KSR1-EGFP to the plasma membrane selected in ROI 3 (see A). C, whole cell analysis of the kinetics of translocation of WT-KSR1-EGFP and DM-KSR1-EGFP to the plasma membrane, as measured by TIRF microscopy. The data were collected at 10-s intervals with interruptions for focus adjustment. DOPA (100 μM) or insulin (100 nM) were added when indicated. The data were obtained from the integrated fluorescence over the surface of the whole cell and normalized to the initial value of this fluorescence.

discrete patches that disappear after several seconds. The mutant KSR1 (EGFP-DM-KSR1) was not mobilized after the addition of DOPA (Fig. 6C, left).

The effects of insulin on the recruitment of KSR1 to the plasma membrane were examined using the same experimental paradigm (Fig. 6C, right). In a manner analogous to that described for the addition of PA, insulin induced a small but significant translocation of EGFP-WT-KSR1 to the plasma membrane while failing to induce EGFP-DM-KSR1 membrane translocation. Like DOPA, insulin-induced KSR1 recruitment was not uniform throughout the cell surface. Furthermore, the effects of insulin required incubation at 37 °C and could not be observed in cells that had been treated at room temperature.

To examine the physiological relevance of the KSR1 patches, HIRcB cells were co-transfected with dsRed2-H-Ras and EGFP-WT-KSR1 and examined by TIRF microscopy. There was a substantial amount of co-localization between H-Ras and KSR1 even in untreated cells (Fig. 7A). This co-localization was mostly observed at the edges of the cell, consistent with accumulation of both proteins in regions enriched in cortical actin. PA and the role of PA in insulin-induced KSR1 translocation were done using the PABR reagents developed in our laboratory. As shown in Table 1, Raf-1-PABR binds PA with high affinity and specificity. Thus, we synthesized a biotinylated derivative of the Raf-1-PABR to be used as a probe to detect the distribution of PA in cultured cells. This biotinylated derivative was tested directly in its ability to bind PA selectively and was found to be indistinguishable from the nonbiotinylated compound. To detect PA, HIRcB cells expressing EGFP-KSR1 and dsRed2-H-Ras were incubated with Raf-1-PABR, which was then detected using Cy5-conjugated streptavidin. Several control assays were done to demonstrate that the binding of the biotinylated PABR was specific for phosphatidic acid. First, we depleted lipids from the cells by washing the coverslips with Triton X-100 after fixing. This treatment eliminated completely the membrane localization of the PABR probe (and reduced significantly the membrane localization of H-Ras), suggesting that the target for PABR is indeed a lipid. Finally, a biotinylated DM-Raf-1-PABR peptide was also synthesized and tested. No binding of the modified peptide was ever observed (Fig. S2).
The results of the experiments performed with the wild type PABR peptide are shown in Fig. 8. In control experiments (cells that had not been stimulated with insulin or DOPA), there was some co-localization of EGFP-KSR1 with both H-Ras or the PABR probe (in the figures shown, the correlation coefficients of the green (KSR1) channel with the red (Ras) and blue (PABR) channels are 0.365 and 0.447, respectively). In contrast, H-Ras and the PABR probe strongly co-localized on the plasma membrane ($R = 0.719$). Short treatments with insulin (100 nM) or DOPA (100 μM) increased the plasma membrane levels of EGFP-KSR1 and induced its co-localization with both H-Ras and the PABR probe ($R = 0.730$ and 0.702, respectively). These data confirm the observations made with live cell TIRF microscopy and provide strong support to the conclusion that H-Ras and KSR1 bind to PA-enriched regions of the cell membrane.

These data suggest a model in which the formation of effective scaffolding complexes during the activation of the ERK cascade requires the binding of PA to specific cellular targets. We have previously reported that Raf-1 is one of these (12, 13), and the work of Zhao et al. (17) showed that the RasGEF Sos is a second important PA target. We demonstrate here that the MEK-ERK scaffolding protein KSR1 also binds PA and that PA binding plays an important role in the modulation of ERK activation by KSR1. Thus, we conclude that PA production plays an important role in the coupling of the ERK cascade signaling module by providing a lipid substrate that will recruit all components of the cascade to a local, PA-enriched microenvironment.

**DISCUSSION**

The primary function of lipid second messengers is the recruitment of specific targets to cellular membranes. This function is often a consequence of specific lipid-protein interactions mediated by well defined lipid binding domains. The specific interactions of phosphatidylinositol derivatives with pleckstrin homology domains have received the most attention, and the specific roles of the interactions of the pleckstrin homology domains of a large number of proteins with inositol 1,4,5-bisphosphate or inositol 1,4,5-trisphosphate have been extensively documented (see Ref. 38 for a review). In recent years, several other “minor” lipid second messengers have been shown to play critical roles in the coupling of important signaling cascades. One of the best examples of this new wave of lipid second messengers is phosphatidic acid.

Our data show that the addition of exogenous DOPA is sufficient to activate the ERK cascade. This result must be interpreted with caution. Exogenous PA may be metabolized to diacylglycerol by the action of PA-phosphohydrolases (29) or to lyso-PA by the action of phospholipase A-type enzymes (31). Diacylglycerol and lyso-PA lead to the activation of the ERK cascade via PKC or the activation of lyso-PA receptors, respectively. Because we did not control for these indirect effects, we cannot conclude that the binding of PA to Raf-1 and KSR1 is sufficient for the activation of the ERK cascade. The data only show that PA formation is necessary. This conclusion is supported by several lines of evidence: (a) PA promotes the association of Raf-1 to the membrane, which is required for the activation of Raf kinases by Ras (10–13); (b) Ras activation by Sos requires PLD2-generated PA (17); and (c) PA modulates KSR membrane binding (this work).

The studies reported here demonstrate that the structural information required to bind PA is contained within a very short sequence of 30 amino acids or less. The PABR sequence interactions with PA with relatively high affinity and remarkable specificity. The PABRs of both KSR1 and Raf-1 bind long chain PAs with an affinity that is at least 10-fold higher than that measured for other anionic phospholipids, such as phosphatidylyserine (Table 1). All of our binding data were nicely fit by
one-site binding isotherms, although the polybasic motifs contained 2–3 positive charges. It has been recently suggested that PA, which is normally a monoaionic phospholipid, loses a second proton upon interaction with the polybasic motif of Raf-1 and becomes dianionic (39, 40). If these structural and computational studies are correct, there is little room (and not enough charge) in the polybasic core of the PABR to promote the binding of more than one PA head group. A second interesting feature of the interactions of PABRs with PA is the fact that Raf-1-PABR binds DOPA, palmityloxy-oleoyl phosphatidic acid, dipalmitoyle-PA, or C6PA with much greater affinity than KSR1-PABR. Our data show that this is due at least in part to the fact that the polybasic core of the Raf-1-PABR (RKTRH) has one additional charge when compared with the KSR1-PABR (RQTRH). This additional charge increases the local charge density, which appears to be critical for high affinity PA binding (39, 40).

Circular dichroism experiments confirmed that the binding of PA to the PABR induces significant conformational changes. The PABR peptides underwent a transition from a random coil conformation to a parallel β-sheet-like structure. No evidence of helical conformations was observed. This is consistent with the data of Takahashi et al. (27), who described the formation of β-sheet structures upon interaction of poly-cationic peptides with PA vesicles. Furthermore, the recently published x-ray crystal structure of the kinase domain of B-Raf (41) shows that the C-terminal half of the PABR of B-Raf, whose sequence is almost identical to that of Raf-1, is mostly structured as a β-sheet. Because binding of PA promotes a transition from a random coil to a β-sheet-like conformation of the PABR, this result suggests that PA stabilizes a conformation of the peptide that resembles its state in the folded protein.

The x-ray structure of the putative kinase domain of KSR1 has not been solved, but our data would predict similar conclusions.

We have shown previously that the translocation of Raf-1 to the plasma membrane is very strongly dependent on the generation of PA. The picture is somewhat different in the case of KSR1. The effects of the addition of PA or insulin treatment on the net levels of membrane-bound KSR1 were modest. Some KSR1-EGFP was found at the plasma membrane of untreated cells, and the addition of PA or insulin increased plasma membrane levels by 30–40%. The smaller effects of PA on KSR1 redistribution are probably a consequence of the lower affinity of KSR1 for PA. Furthermore, KSR1 has been shown to bind phosphatidylinerse via the cysteine-rich domain located near its N terminus (23), which may explain the partial membrane localization of KSR1 in naive cells. We propose that the binding of KSR1 to membranes is mediated by several distinct and probably independent interactions of medium affinity (probably in the micromolar range), which cooperate to promote the translocation of KSR1 to the membrane. Importantly, the distribution of DM-KSR1 was not altered by the addition of either PA or insulin. Therefore, we conclude that the effects of PA and insulin on the translocation of KSR1 to the plasma membrane are a consequence of the direct interaction of KSR1 with PA.

An important observation is that the redistribution of EGFP-KSR1 after treatment with PA or insulin was not uniform throughout the membrane. In fact, quantitative analysis of the TIRF data demonstrated that the effects of PA and insulin were localized and that KSR1-EGFP redistributed to "patches" concentrated in some regions of the plasma membrane (Fig. 6C and Movie S1). This behavior was very similar to that of a dsRed2-H-Ras construct. In fact, when the KSR1 and H-Ras constructs were imaged simultaneously, the addition of PA or insulin induced the segregation of both proteins into the same structures.

It should be noted that the addition of exogenous PA cannot be compared directly with growth factor receptor stimulation. Growth factors increase the membrane levels of PA by a small factor. Insulin, for instance, produces a 2-fold increase, which is sustained for at least 20 min in HIRcB cells (12). However, the effects of exogenous PA and insulin on KSR1 (and Raf-1 (see Ref. 12)) translocation are comparable. How is it, then, that small changes in the levels of PA have such dramatic effects in...
the activation of the ERK cascade? The answer to this question is probably 2-fold. First, at least three of the elements of the ERK cascade (Sos, Raf-1, and KSR1) are recruited to membranes by PA. Assuming a simple 1:1 stoichiometry, this implies at least three molecules of PA. The probability of the formation of a complex involving these components increases with the third power of the concentration of PA (i.e., a doubling of the levels of PA increases the probability of the formation of the complex by a factor of 8). Second, several lines of evidence suggest that PA has a tendency to segregate into PA-rich domains (42–45). In fact, molecular dynamics simulations of the structure of PA in model membranes suggest that PA may form a separate, ordered phase in the presence of small concentrations of divalent cations (45). Our data lend support to this idea, providing direct evidence of the existence of PA-rich clusters in cell membranes (Fig. 8). Furthermore, these data clearly indicate that PA, KSR1, and H-Ras are concentrated in the same regions of the cell membrane. Thus, the general model that emerges from these studies is the following: activation of the insulin receptor (and probably others as well) produces a local pool of PA, structured in relatively stable, separated microdomains, that act as true molecular scaffolds in the coupling of the ERK cascade.

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