Identification of Key Residues in the A-Raf Kinase Important for Phosphoinositide Lipid Binding Specificity

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ABSTRACT: Raf kinases are involved in regulating cellular signal transduction pathways in response to a wide variety of external stimuli. Upstream signals generate activated Ras-GTP, important for the relocalization of Raf kinases to the membrane. Upon full activation, Raf kinases phosphorylate and activate downstream kinase in the mitogen-activated protein kinase (MAPK) signaling pathway. The Raf family of kinases has three members, Raf-1, B-Raf, and A-Raf. The ability of Raf-1 and B-Raf to bind phosphatidylserine (PS) and phosphatidic acid (PA) has been show to facilitate Raf membrane associations and regulate Raf kinase activity. We have characterized the lipid binding properties of A-Raf, as well as further characterized those of Raf-1. Both A-Raf and Raf-1 were found to bind to 3-, 4-, and 5-monophosphorylated phosphoinositides [PI(3)P, PI(4)P, and PI(5)P] as well as phosphatidylinositol 3,5-bisphosphate [PI(3,5)P_2]. In addition, A-Raf also bound specifically to phosphatidylinositol 4,5- and 3,4-bisphosphates [PI(4,5)P_2 and PI(3,4)P_2] and to PA. A mutational analysis of A-Raf localized the PI(4,5)P_2 binding site to two basic residues (K50 and R52) within the Ras binding domain. Additionally, an A-Raf mutant lacking the first 199 residues [i.e., the entire conserved region 1 (CR1) domain] bound the same phospholipids as full-length Raf-1. This suggests that a second region of A-Raf between amino acids 200 and 606 was responsible for interactions with the monophosphorylated PIs and PI(3,5)P_2. These results raise the possibility that Raf-1 and A-Raf bind to specific phosphoinositides as a mechanism to localize them to particular membrane microdomains rich in these phospholipids. Moreover, the differences in their lipid binding profiles could contribute to their proposed isoform-specific Raf functions.

Raf kinases are important regulators of signal transduction pathways that control cell growth, differentiation, and apoptosis. In response to an upstream stimulus such as activation of a growth factor receptor, the Ras GTP-binding protein recruits Raf kinases to the plasma membrane. After a series of activating phosphorylation events, Raf kinases can phosphorylate and activate mitogen-activated protein kinase (MAPK)[1]/extracellular regulated kinase (ERK) kinases (MEK) and the downstream MAPK pathway.

Raf kinases can be phosphorylated by numerous kinases, resulting in complex regulatory mechanisms that are not fully understood (reviewed in refs *1*−*5*). Adding to this complexity, the phosphorylation of different Raf family members by the same kinase can have opposing effects on Raf kinase activity. While Raf family members share a common ability to activate the MEK/MAPK pathway, there is increasing evidence to suggest that they also possess unique isoform-specific functions, including interactions with different proteins (G proteins, scaffolds, chaperones, adapters, kinases, substrates, and regulatory proteins) and diverse subcellular localizations (*1*−*5*).

There are three members of the Raf family of serine/threonine kinases, Raf-1 (also known as c-Raf), A-Raf, and B-Raf. Each of the Raf kinases contains three conserved regions: CR1, CR2, and CR3. CR1 is the most N-terminal and this region includes a Ras binding domain (RBD) as well as cysteine-rich domain (CRD) capable of binding two molecules of zinc. CR2 is a small region containing many

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[1] Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular regulated kinase; MEK, mitogen-activated protein/extracellular regulated kinase; CR1, CR2, and CR3, conserved regions 1, 2, and 3; RBD, Ras binding domain; CRD, cysteine-rich region; PS, phosphatidylserine; PA, phosphatidic acid; PI, phosphoinositide; PI(3)P, phosphatidylinositol 3-phosphate; PI(4)P, phosphatidylinositol 4-phosphate; PI(5)P, phosphatidylinositol 5-phosphate; PI(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P_2, phosphatidylinositol 3,5-bisphosphate; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; LPA, lysophosphatidic acid; LPC, lysophosphocholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S1P, sphingosine 1-phosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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regulatory phosphorylation sites. CR3 is the C-terminal serine/threonine kinase domain, the activity of which can be regulated by the other two conserved regions (*3*, *6*).

The lipid binding activity of Raf-1 has been described to include an ability to interact with phosphatidylserine (PS) via the CRD, as well as phosphatidic acid (PA) in the C-terminal catalytic domain (*7*−*11*). Raf-1 has also been demonstrated to interact with ceramide, likely via the CRD, albeit the strength of this interaction is secondary to that of Raf-1 with PA and PS (*12*). The structure of the CRD of Raf-1 has been determined (*13*) and compared to a similar CRD domain in protein kinase C capable of binding PS, phorbol esters and diacylglycerol (*14*). Membrane phospholipids have been shown to enhance the activity of Raf-1 (*15*), as well as assisting in membrane association of the Raf-1 kinase (*12*, *16*). The importance of the lipid binding properties of Raf-1 is illustrated by the fact that zebrafish embryos expressing a mutant Raf-1 unable to bind PA developed abnormal bent trunk and tail structures (*17*).

Purified B-Raf exhibits lipid binding properties similar to those of Raf-1 (*12*), and PS was found to enhance B-Raf activity, in a K-Ras dependent manner (*18*). To date no lipid binding activity has been reported for A-Raf. Therefore, experiments were undertaken to characterize the lipid binding activity of A-Raf and potentially delineate interactions specific to this family member.

In this paper, we report that both A-Raf and Raf-1 bind to the monophosphorylated phosphoinositide (PI) lipids PI(3)P, PI(4)P, and PI(5)P and to bisphosphorylated PI(3,5)P_2. Uniquely, the A-Raf kinase also binds more of the multiply phosphorylated lipids and its kinase activity is negatively regulated by both PI(4,5)P_2 and PI(3,4,5)P_3. Analysis of the lipid binding properties of a series of A-Raf mutants has identified residues that are necessary for PI(4,5)P_2 binding, as well as other residues that are critical in determining the phospholipid binding selectivity of A-Raf. The interactions between A-Raf and specific phospholipids may regulate not only the activity of the kinase but also its association with specific membranes within the cell.

MATERIALS AND METHODS

Generation and Expression of Wild-Type and Mutant A-Raf (and Raf-1) Proteins. The full-length wild-type human Raf-1 cDNA (encoding amino acids 1−648) was amplified by polymerase chain reaction with plasmid pECE-c-Raf (gift from Dr. T. Pawson, Samuel Lunenfeld Research Institute, Toronto, ON) as a template. The product was digested with *Bam*HI and *Eco*RI and ligated into similarly digested pGEX6P1 (Amersham Pharmacia). The full-length wild-type human A-Raf cDNA (encoding amino acids 1−606) and a truncated portion of A-Raf (CR2−CR3; amino acids 200−606) were amplified similarly, with the HA-A-Raf plasmid (*19*) as a template. Each was also subcloned into the *Bam*HI and *Eco*RI sites of pGEX6P1. The entire coding sequence of each was verified by DNA sequencing to ensure that no mutations were present. The A-Raf basic-X-basic mutants have been described previously (*19*) and were full-length human A-Raf sequences encoding the following mutations of basic residues to alanine: site A (K28A and R30A), site B (K50A and R52A), site C (K66A and R68A), site R (R209A and R211A), site D (R279A and R281A), site E (K288A, K289A, K290A, and K292A), site F (R326A and R328A), site G (K360A and R362A), and site H (K454A and R456A). The A-Raf DNA containing each mutation was subcloned from the HA-A-Raf vectors (*19*) into pGEX6P1, similar to that described above. Each mutant was verified by DNA sequencing to contain the expected A-Raf mutations in the resulting pGEX6P1-A-Raf mutant plasmids.

For protein expression, each plasmid was expressed in *Escherichia coli* strain BL21 (Novagen) and proteins were induced and purified essentially according to previously described methods (*7*, *20*). Briefly, cultures were initially grown at 37 °C to an absorbance of 0.5 (600 nm). Zinc chloride (to 2 μM) was added and protein expression was induced for 1−3 h by use of isopropyl β-D-thiogalactopyranoside (to 0.1 mM) at 20 °C. Bacteria were lysed by sonication and bound to glutathione−Sepharose beads (Amersham Pharmacia), according to the supplier's instructions. Samples were cleaved from the immobilized GST portion by incubation with PreScission protease (Amersham Pharmacia). In most instances, full-length Raf proteins were further purified from proteolyzed protein fragments by gel-filtration chromatography. Samples were concentrated by use of an Amicon ultracentrifugal filter (molecular weight cutoff 30 000), and buffer was exchanged into protein storage buffer (100 mM $NaHCO_3$, 100 mM NaCl, and 1 mM dithiothreitol). The purity of each wild-type protein was at least 90%, as assessed by SDS−PAGE and Coomassie Blue staining, as well as immunoblotting with anti-Raf-1 or anti-A-Raf antibodies (*19*). Aliquots were flash-frozen and stored at −80 °C. Prestained markers were obtained from Sigma (SDS-7B).

Protein−*Lipid Overlay Assay*. Nitrocellulose-immobilized phospholipids (100 pmol/spot, PIP-Strips; Echelon Biosciences Inc.) were lysophosphatidic acid (LPA), lysophosphocholine (LPC), D-*myo*-phosphatidylinositol (PI), D-*myo*-phosphatidylinositol 3-phosphate [PI(3)P], D-*myo*-phosphatidylinositol 4- phosphate [PI(4)P], D-*myo*-phosphatidylinositol 5-phosphate [PI(5)P], L-α-phosphatidylethanolamine (PE), L-α-phosphatidylcholine (PC), sphingosine 1-phosphate (S1P), D-*myo*-phosphatidylinositol 3,4-bisphosphate [PI(3,4,)-P_2], D-*myo*-phosphatidylinositol 3,5-bisphosphate [PI(3,5,)-P_2], D-*myo*-phosphatidylinositol 4,5-bisphosphate [PI(4,5,)P_2], D-*myo*-phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3], L-α-phosphatidic acid (PA), and L-α-phosphatidylserine (PS). The nitrocellulose lipid blots were incubated in blocking buffer [3% (w/v) fatty acid-free bovine serum albumin (Sigma) in TBST: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% (v/v) Tween-20] for 1 h at 20 °C. The blots were then incubated overnight at 4 °C in 0.5 μg/mL purified Raf-1 wild type, A-Raf wild type, or A-Raf mutant protein. Lipid blots were washed in TBST (6 times, 5 min each) and incubated for 1 h at 20 °C with the corresponding antibody (1 μg/mL in blocking buffer) to either Raf-1 (Santa Cruz Biotechnology, sc-133) or A-Raf (Santa Cruz Biotechnology, sc-408). Blots were washed as before and incubated for 1 h at 20 °C with anti-rabbit horseradish peroxidase (0.2 μg/mL in blocking buffer). Raf proteins bound to the phospholipids immobilized on the membrane were visualized by use of Western Lightning chemiluminescence reagent plus (Perkin-Elmer Life Sciences) according to the manufacturer's instructions. Each blot shown is representative of at least three independent experiments, each with a fresh lipid

membrane. A-Raf protein was denatured in 10 mM sodium phosphate monobasic, 1% 2-mercaptoethanol, 1% SDS, and 6 M urea) at 100 °C for 5 min.

A-Raf Kinase Assay. Lipid micelles used in the kinase assays were prepared as follows: PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2, and PI(3,4,5)P_3 (Echelon Biosciences Inc.) were obtained as ammonium salts and made up in distilled water to 1 mg/mL. PC (Sigma) was resuspended in chloroform/methanol (1:1 v/v) to 1 mg/mL. PC (90%) and test lipid (10%) were dried down under nitrogen and resuspended in buffer L (50 mM HEPES, pH 7.2, and 100 mM NaCl) to a concentration of 1 mg/mL. Lipid micelles were generated by sonication for 30 s at a setting of 1 with the microtip of a Branson sonifier 450, after which they were stored at 4 °C for up to 1 week.

A-Raf (3 μg), Raf-1 (3 μg), or activated Raf-1 (0.4 unit; Upstate Biotechnology catalog no. 14-200; lysate from full-length wild-type untagged recombinant human Raf-1 coexpressed in Sf9 cells with Ras and Lck) were assayed in a final volume of 20 μL of kinase buffer (30 mM HEPES, pH 7.4, 7 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 15 μM ATP) containing lipid micelles (5 μg), [γ-^{32}P]ATP (20 μCi), and GST-MEK1-His (unactive; 2 μg, Upstate Biotechnology catalog no. 14-205) for 20 min at 20 °C. The reactions were resolved by SDS−PAGE (10%), and Coomassie stained gels (*19*) were imaged by use of a Bio-Rad gel documentation system and Quantity One software. Gels were dried, visualized by autoradiography, and quantified by use of a phosphorimager (Bio-Rad Laboratories) and Quantity One software. The autophosphorylation observed in the GST-MEK alone lane was subtracted from the total observed phosphorylation of GST-MEK to obtain the phosphorylation by A-Raf. Statistical analysis was performed with Prism software (GraphPad Software, Inc., San Diego, CA).

RESULTS

A-Raf Binds to Phosphatidylinositides, Some of Which Negatively Regulate A-Raf Kinase Activity. To characterize the lipid binding properties of A-Raf, bacterially expressed and purified full-length wild-type A-Raf and Raf-1 were prepared (Figure 1a, upper panel). These purified A-Raf and Raf-1 proteins were able to phosphorylate a GST-MEK substrate, though not as well as an activated Raf-1 that had been coexpressed in Sf9 cells with Ras and Lck (Figure 1a, lower panel). The A-Raf protein was selectively recognized by anti-A-Raf antibodies (Figure 1b), while the Raf-1 protein was specifically recognized by anti-Raf-1 antibodies (Figure 1c).

Raf-1 and A-Raf were assessed for their ability to bind to a variety of lipids bound to nitrocellulose by a protein−lipid overlay assay. Nitrocellulose lipid blots that contained 100 pmol/spot of lysophosphatidic acid (LPA), lysophosphocholine (LPC), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylserine (PS), PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2, PI(3,4,5)P_3, or sphingosine 1-phosphate (S1P) were probed with purified A-Raf or Raf-1 protein solutions followed by the corresponding specific anti-Raf antibodies (Figure 1d−f). The results from these protein−lipid blots demonstrate that both kinases can bind to PI(3)P, PI(4)P, and PI(5)P, the monophosphorylated forms of PI, as well as to PI(3,5)P_2 (Figure 1e,f). A-Raf also bound well to PI(4,5)P_2 and PA, with lesser amounts bound to PI(3,4)P_2. Minimal amounts of A-Raf bound to PI, PI(3,4,5)P_3, and PS. As compared to A-Raf, Raf-1 bound noticeably less to PI(4,5)P_2 and possibly PA and PI(3,4)P_2. For both A-Raf and Raf-1, no detectable binding was observed to LPA, LPC, PE, PC, and S1P.

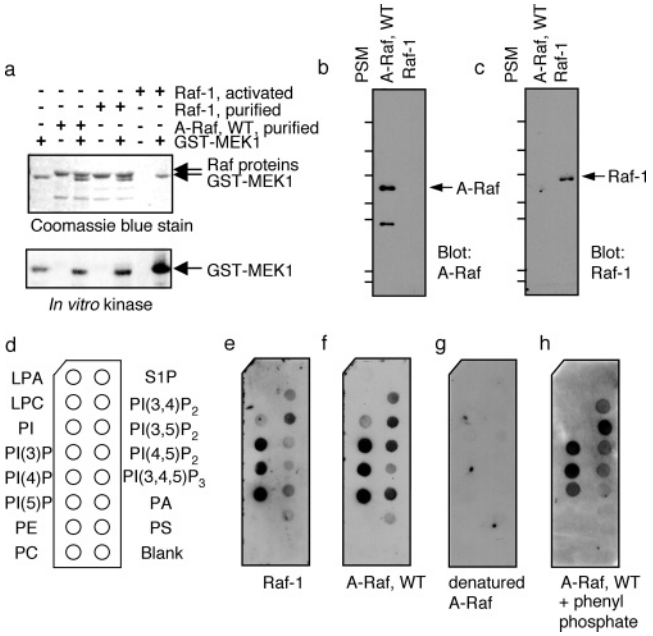

FIGURE 1: Both Raf-1 and A-Raf bind to phosphoinositides. Raf-1 and A-Raf were purified as GST fusion proteins and liberated from the GST portions by PreScission cleavage. (a) Purified proteins were resolved by SDS−PAGE (10%) and stained with Coomassie blue (upper panel). The ability of these purified A-Raf and Raf-1 proteins to phosphorylate a GST-MEK1 substrate was also determined by an in vitro kinase assay (lower panel). These protein preparations were also tested for their immunoreactivity toward anti-A-Raf specific (b) and anti-Raf-1-specific (c) antibodies, by an immunoblot analysis. The prestained markers (PSM) migrate at the apparent molecular masses of 172.5, 112.5, 86, 62.5, 53, 33.5, and 31.5 kDa (top to bottom). Nitrocellulose membranes containing the indicated lipids (d) (100 pmol/spot) were blocked and probed with full-length purified Raf-1 protein (e) or A-Raf protein (f). Bound proteins were detected with rabbit antibodies specific for the respective Raf protein, followed by anti-rabbit horseradish peroxidase-conjugated secondary antibodies, and visualized by chemiluminescence. Each blot shown is representative of at least three independent experiments, each using a fresh lipid membrane. (g) Denatured A-Raf was used to probe the lipid blot and was detected as for the native wild-type A-Raf protein in panel f. (h) The lipid blot was probed with A-Raf protein in the presence of phenyl phosphate (500 nM). LPA = lysophosphatidic acid; LPC = lysophosphocholine; PI = phosphatidylinositol; PI(3)P = phosphatidylinositol 3-phosphate; PI(4)P = phosphatidylinositol 4-phosphate; PI(5)P = phosphatidylinositol 5-phosphate; PE = phosphatidylethanolamine; PC = phosphatidylcholine; S1P = sphingosine 1-phosphate; PI(3,4)P_2 = phosphatidylinositol 3,4-bisphosphate; PI(3,5)P_2 = phosphatidylinositol 3,5-bisphosphate; PI(4,5)P_2 = phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P_3 = phosphatidylinositol 3,4,5-trisphosphate; PA = phosphatidic acid; PS = phosphatidylserine.

We also probed a lipid blot with denatured A-Raf protein (Figure 1g) and observed no binding, indicating that the folded structure of A-Raf was required for lipid binding. To determine if increased charged phosphate groups could inhibit A-Raf binding to the phosphoinositide lipids, we probed a lipid blot with A-Raf protein in the presence of

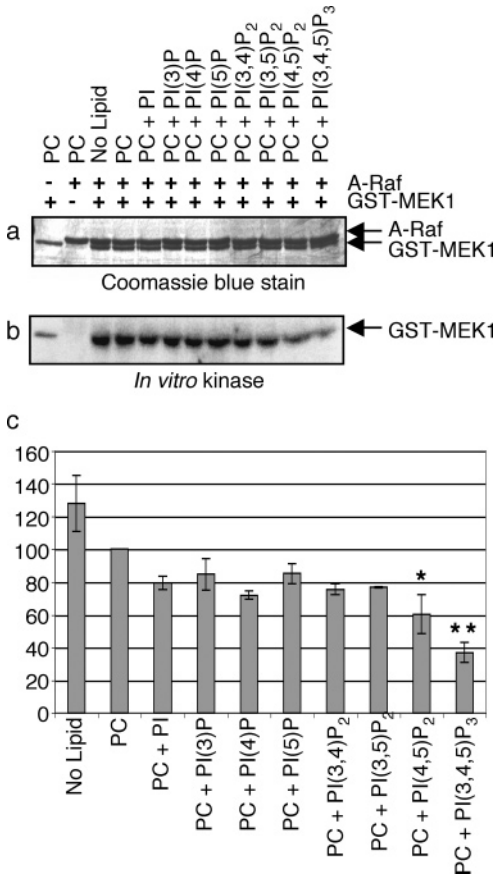

FIGURE 2: Phospholipids reduce A-Raf kinase activity toward MEK1. (a) Lipid micelles containing PC (90%) and the indicated test lipid (10%) were included in an A-Raf kinase assay with [γ-^{32}P]-ATP, with GST-MEK1 as a substrate. Kinase reactions were resolved by SDS−PAGE, and gels were stained with Coomassie blue. (b) Gels were dried and phosphorylated GST-MEK1 was visualized using autoradiography. One typical result is shown. (c) Results from three independent kinase assays were quantified by use of a phosphorimager and Quantity One software. The mean ± SEM is shown. The statistical analysis was performed with Prism software to compare the A-Raf kinase activity in the presence of each test lipid as compared to that in the presence of PC alone. Two samples were statistically significantly different: *, $p < 0.01$; **, $p < 0.001$.

phenyl phosphate (500 nM) (Figure 1h). A-Raf bound to similar phosphoinositides in the presence of phenyl phosphate but bound notably less well to PA.

In addition to the well-characterized interactions of Raf-1 and B-Raf with PA and PS, these results provide the first description of an association between each of A-Raf and Raf-1 with various phosphatidylinositides. Further, the differential lipid binding properties of A-Raf and Raf-1 could provide a mechanism to specifically regulate the activity and/or localization of these cellular kinases through selective interactions with different phosphorylated forms of these phosphoinositide lipids.

Previous reports have indicated that lipid binding by Raf family kinases can regulate their kinase activity. Thus, A-Raf kinase activity toward the GST-MEK protein was assayed in the presence of micelles consisting of different lipid compositions. After incubation, the entire kinase reaction was resolved by SDS−PAGE (Figure 2a) and the phosphorylated proteins were visualized by autoradiography (Figure 2b) and quantified by use of a phosphorimager (Figure 2c). Each of the test micelles was composed of 90% PC and 10% test lipid, thus their individual effects on the activity of A-Raf were analyzed in comparison to results obtained with 100% PC. The micelles were composed of 90% PC (or 100% PC as a control), as this lipid was not observed to bind to A-Raf (Figure 1f) and did not significantly alter the activity of this kinase (Figure 2). We observed a small amount of autophosphorylation activity for the GST-MEK protein in the absence of A-Raf. Statistical analysis by one-way ANOVA and Tukey's multiple comparison indicate that most test lipids [PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, and PI(3,5,)P_2] did not have a significant effect on the kinase activity of A-Raf toward MEK1 ($p > 0.05$). In contrast, PI(3,4,5)P_3 and PI(4,5)P_2 reduced A-Raf kinase activity to 38% ($p < 0.001$) and 60% ($p < 0.01$), respectively, when compared to A-Raf activity in the presence of PC alone. To determine if increasing the concentration of negatively charged phosphate groups around A-Raf was sufficient to inhibit its kinase activity in the absence of phosphoinositides, we assayed A-Raf activity in the presence of PC and phenyl phosphate (50 nM). Under these conditions, A-Raf kinase activity actually increased and was similar to A-Raf activity in the absence of lipid (data not shown). These assays demonstrated that although the previous lipid blot binding data did not show the strongest, or perhaps the most stable, interaction between A-Raf and PI(3,4,5)P_3, this lipid clearly had a significant impact on A-Raf kinase activity. Thus, A-Raf kinase activity can be negatively regulated by PI(4,5)P_2, but even more strongly by the PI3 kinase lipid product PI(3,4,5)P_3.

Identification of A-Raf Residues Important for PI Lipid Binding Specificity. Our next series of experiments focused on characterizing the residues in A-Raf responsible for lipid binding specificity. A previous report has shown that PI(4,5)P_2 lipids bound to the sequence **K**LFQV**K**G**RR**, and likely to a second region containing the sequence **K**S-GL**KY**K**K**, within the gelsolin protein (*21*). These authors suggested that binding to PI(4,5)P_2 was mediated by a consensus basic amino acid motif of B-X_{3-5}-B-X-B-B (where B = basic residues R or K and X = any residue). Another report showed that the C-terminal SH2 domain of p85, the regulatory subunit of PI3 kinase, bound to PI(3,4,5)P_3 lipids via a similar sequence, **R**N**K**AENLL**R**G**KR** (amino acids 631−642) (*22*).

Both of these consensus phosphoinositide binding sequences contained at least one core BXB sequence motif. We searched the A-Raf sequence for similar BXB sequences and identified nine such regions throughout the A-Raf sequence that were previously designated as sites A, B, C, R, D, E, F, G, and H [Figure 3a; (*19*)]. Mutant A-Raf proteins in which both of the basic residues had been mutated to alanine at each of these sites were purified (Figure 4a) and tested for their immunoreactivity toward anti-A-Raf antibodies (Figure 4b). We also tested a truncated A-Raf protein (CR2−CR3; amino acids 200−606) that lacked the entire CR1 domain and so was missing both the RBD and the CRD (Figures 3 and 4). Each protein preparation was found to be 50−90% pure and retained its ability to be recognized by the C-terminal-specific anti-A-Raf antibodies.

The lipid binding specificities of each mutant A-Raf protein was tested by a protein−lipid overlay assay (Figure 5), the results of which are summarized in Figure 3. Mutation of sites C and R actually increased binding to PI lipids

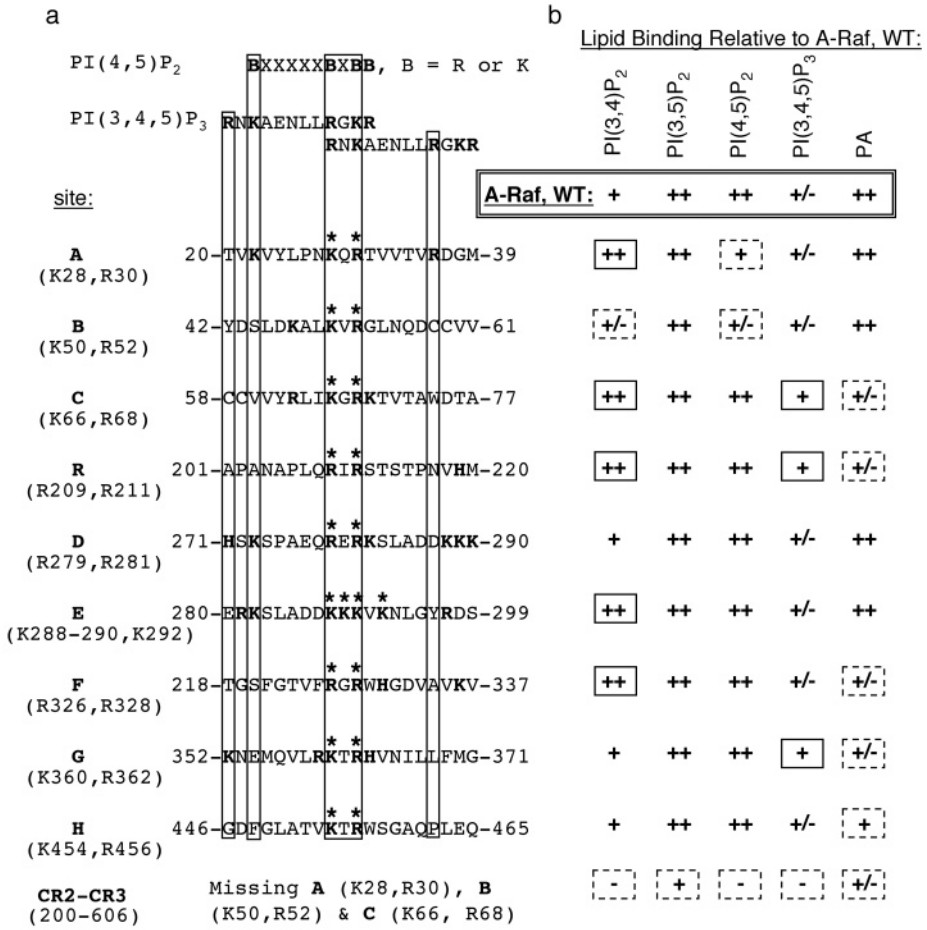

FIGURE 3: Basic regions within A-Raf compared to known phosphoinositide binding sequences. (a) Sequence alignment between previously characterized PI(4,5)P_2 and PI(3,4,5)P_3 binding sites and regions of A-Raf containing similar basic-X-basic sequences, indicating the designated "site" within A-Raf (where basic = B, arginine (R) or lysine (K), and X = any amino acid). (b) Selected results of the lipid binding abilities of A-Raf proteins containing mutations of the indicated sites as compared to the binding of wild-type A-Raf (A-Raf, WT) shown at the top. The representative lipid blot results are shown in Figure 5. Observed binding between the A-Raf mutants and selected lipids (listed across the top): ++ (significant binding); + (binding); ± (minimal binding); − (no binding). Changes in binding for the mutant A-Raf as compared to the A-Raf, WT protein are indicated as follows: solid boxes indicate increased binding, while dotted boxes indicate decreased binding.

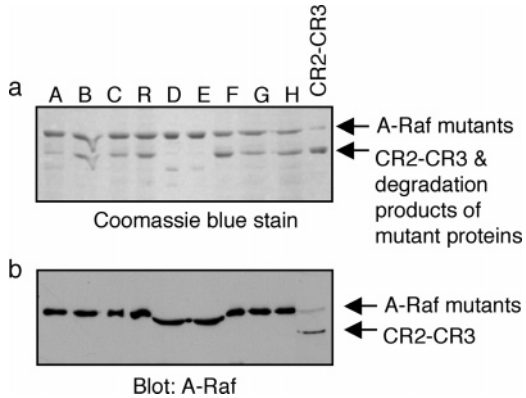

FIGURE 4: Purification and immunoreactivity of A-Raf mutants. (a) Purified A-Raf mutant proteins were resolved by SDS−PAGE and stained with Coomassie blue. The upper arrow indicates the size of the full-length A-Raf proteins for all mutants except the CR2−CR3. The lower arrow indicates the location of the intact CR2−CR3 protein, as well as proteolytic breakdown products of the other mutant proteins. (b) Immunoblot analysis of mutant A-Raf proteins probed with anti-A-Raf-specific antibodies.

phosphorylated at the 3,4 positions, suggesting that these residues are important in maintaining the lipid binding preferences within the wild-type A-Raf protein, in particular, in reducing the binding to PI(3,4)P_2 and PI(3,4,5)P_3. Mutations within the A-Raf kinase domain (sites F, G, and H) all show reduced binding to PA. This is not surprising for site G within A-Raf, since an identical sequence within Raf-1 has previously been shown to be required for interactions with PA (*8*). Mutations within sites A and E had small effects, both increasing A-Raf binding to PI(3,4)P_2, while mutation of site D had little or no effect on the lipid binding properties of A-Raf. These latter results were somewhat unexpected since sites A, D, and E most closely resemble the previously reported consensus binding sequence for PI(4,5)P_2 (Figure 3). The CR2−CR3 mutant and the site B mutant of A-Raf showed the most decreases in polyphosphoinositide binding, with little or no binding to PI(4,5)P_2 (Figure 5b), and most closely resembled the lipid blots probed with Raf-1 (Figure 1e). None of these A-Raf mutations or deletions prevented interactions with the monophosphorylated PIs or with PI(3,5)P_2. Thus, the basic residues in site B (K50, R52) of A-Raf are important for determining lipid binding specificity toward PI(4,5)P_2. This suggests that A-Raf contains an additional PI binding sequence within its CR2−CR3 domains (residues 200−606), and since Raf-1 binds similar phospholipids, it likely does as well.

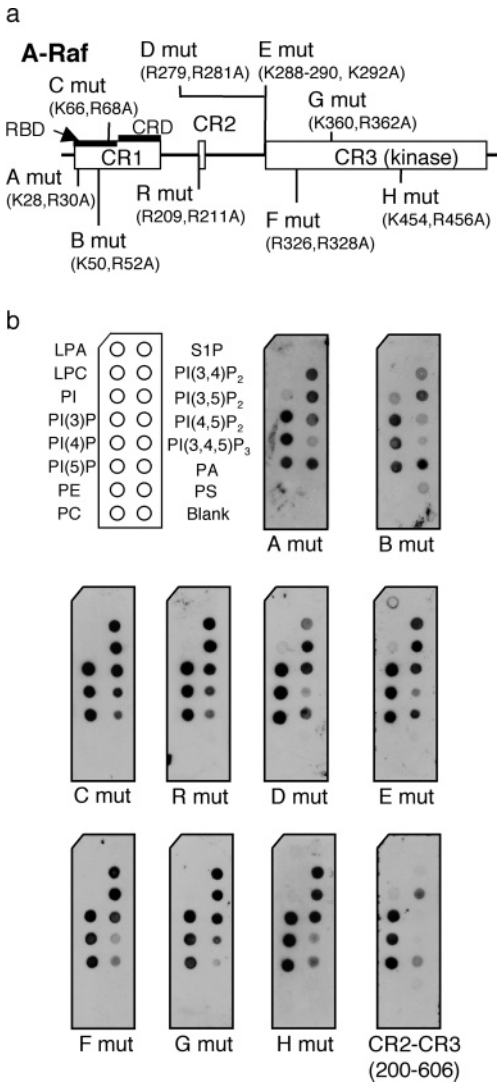

FIGURE 5: Lipid binding properties of A-Raf mutants. (a) Schematic diagram of the domain structure of A-Raf and the locations of the different basic-X-basic sites designated A, B, C, R, D, E, F, G and H. (b) Indicated mutant A-Raf proteins in which both basic residues had been changed to alanines (or for site E, four basic residues K288, K289, K290, and K292 were all changed to alanines) were used in a protein−lipid overlay assay as described in Figure 1. Bound mutant A-Raf proteins were detected as described previously by use of anti-A-Raf antibodies. CR1, CR2, and CR3 = conserved regions 1, 2, and 3; RBD = Ras binding domain; CRD = cysteine-rich domain.

DISCUSSION

This is the first report to show that both A-Raf and Raf-1 kinases bind to phosphoinositide lipids, important mediators of protein localization and potential regulators of protein activity. Both A-Raf and Raf-1 bound to the monophosphorylated phosphoinositides PI(3)P, PI(4)P, and PI(5)P as well as to $PI(3,5)P_2$. Additionally, the A-Raf kinase bound well to $PI(4,5)P_2$ and PA and somewhat to $PI(3,4)P_2$. The kinase activity of A-Raf was also found to be negatively regulated by $PI(4,5)P_2$, and $PI(3,4,5)P_3$, the latter of which it binds to relatively poorly. These results suggest that strong binding by A-Raf to $PI(3,4,5)P_3$ is not a prerequisite for inhibition of A-Raf kinase activity. In addition, lipid−A-Raf interactions may serve two distinct functions: localization of A-Raf to specific membrane microdomains and regulation of A-Raf kinase activity. Moreover, since A-Raf can bind selectively to some phosphoinositides, most notably $PI(4,5)P_2$, that Raf-1 cannot, it could provide a mechanism to differentially localize A-Raf to specific membranes enriched in these phospholipids.

Phosphoinositides are associated with specific membrane locations, and their best described functions are in cell signaling and protein trafficking pathways (*23*). Both $PI(3,4)P_2$ and $PI(3,4,5)P_3$ are important second messengers generated by PI3 kinase at the plasma membrane in response to growth factor stimulation. They function to relocalize PDK-1 and Akt to the membrane, a critical step for their activation and subsequent anti-apoptotic signaling functions (*24*). $PI(4,5)P_2$ is found primarily at the plasma membrane and is a substrate for PI3 kinase as well as for phospholipase Cγ1-mediated hydrolysis and the generation of diacylglyercol and inositol trisphosphate, important for protein kinase C and Ca^{2+} signaling, respectively (*25*). Since phosphoinositides also serve as substrates for PI kinases, PI phosphatases, and lipases, their structures can be altered to modulate these binding and regulatory functions during complex sequential processes such as cell signaling and endocytosis.

$PI(4,5)P_2$ is also required for the formation of clathrin-coated pits during endocytosis. PI(3)P is found on endocytic vesicles and it targets PI(3)P binding proteins to these sites, where they function in membrane fusion events in the endocytic pathway. $PI(3,5)P_2$ has been suggested to localize to late endosomes and lysosomes, vesicles important at later steps in the endocytic process. PI(4)P functions on Golgi membranes in vesicle trafficking, while PI(5)P is present in the nucleus and may be involved in cellular responses to DNA damage (*26*).

Phosphoinositides play important roles during cell signaling and vesicle trafficking by creating membrane microdomains and binding proteins with lipid-specific interaction domains (e.g., FYVE, ENTH, PX, and PH). A few of these lipid binding domains are highly selective and only bind one PI, but most can accommodate several related PI lipids (*27*). Where structural data is available, this broad specificity has been shown to result from fewer interactions between the protein's lipid binding pocket and the lipid phosphates. This broad specificity has been suggested to provide a mechanism for a more sustained interaction between the protein and the lipid.

The low degree of sequence conservation present in some of these domains (e.g., PH) can make it difficult to be certain that a particular lipid binding domain is present and functional on the basis of protein sequence data alone. This is likely because frequently only a small number of residues participate directly in lipid binding. Small sequence motifs, typically containing a cluster of basic residues, can also mediate binding to lipids such as $PI(4,5)P_2$ and $PI(3,4,5)P_3$ as detailed previously (*21*, *22*). Raf kinases do not appear to contain any PH, PX, ENTH, or FYVE domains, though they do have a CRD involved in binding PS (*10*). Although the cysteine-rich domain forms a conserved binding fold that incorporates two molecules of zinc, only a few basic residues (R143, K144, and K148) are involved in binding PS. Similarly, a small basic sequence motif (398-RKTRH-402) is responsible for binding to PA within the kinase domain of Raf-1 (*8*).

Some lipid binding domains interact with the phospholipid on the surface of the domain, rather than by forming a

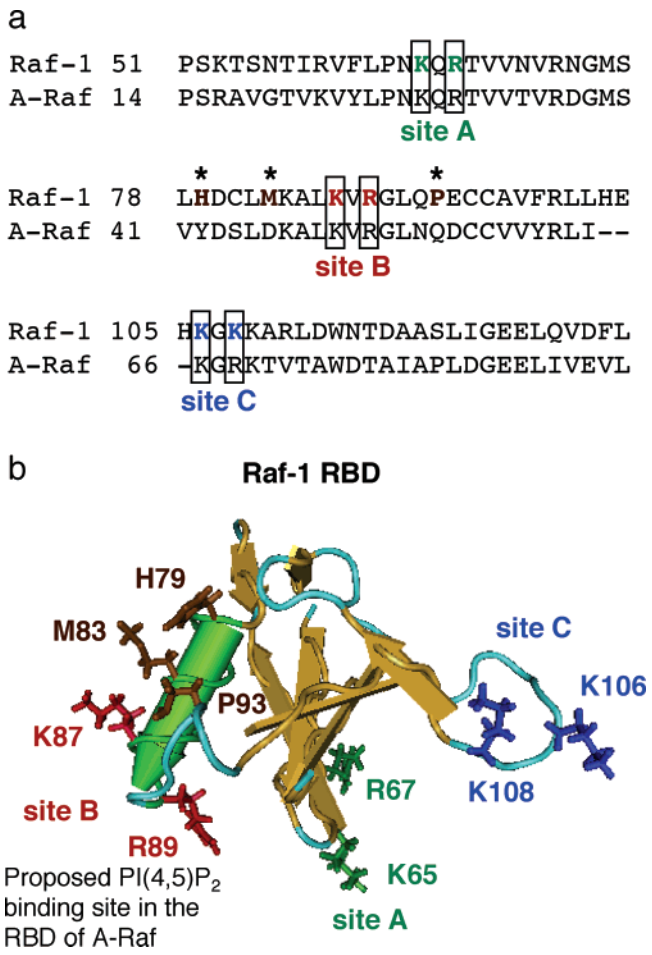

FIGURE 6: Ras binding domain (RBD) of A-Raf contains the site B basic residues required for $PI(4,5)P_2$ binding. (a) Sequence alignment of RBDs of human Raf-1 and human A-Raf. The location of the basic-X-basic sequences at sites A, B, and C are boxed and color-coded to match the structure shown in panel b. In addition, three residues that are poorly conserved between the Raf-1 and A-Raf sequences, flanking the basic residues in site B, are highlighted with asterisks. (b) Structure of the Raf-1 RBD (*29*). The three-dimensional structure was displayed and the residues of interest were featured by use of Cn3D version 4.1 (www.ncbi.nim.nih.gov). Site A (Raf-1, K65 and R67) are dark green; site C (Raf-1, K106 and K108) are dark blue; site B (Raf-1, K87 and R89) are red, with flanking residues not conserved between Raf-1 (H79, M83, and P93) and A-Raf shown in brown. The corresponding nonconserved A-Raf residues are Y42, D46, and Q56.

binding pocket to accommodate the lipid moiety (*27*). For example, the ENTH domain of the CALM protein, involved in clathrin lattice assembly, binds $PI(4,5)P_2$ by use of three lysines and a histidine residue, projected from the surface of the ENTH domain (*28*). While there is no structural information available for A-Raf, the structure of the RBD of Raf-1 (*29*) shows the positions of the two basic residues corresponding to site B [which we show is required for $PI(4,5)P_2$ binding] on A-Raf, at the end of the α-helix (K87 and R89 of Raf-1, Figure 6b). If the analogous region of A-Raf has a related structure, the corresponding basic residues at site B (i.e., K50 and R52 of A-Raf; Figure 6a) would be positioned similarly. A-Raf binds well to $PI(4,5)P_2$ and requires the basic residues at site B for this interaction, yet Raf-1 does not bind well to $PI(4,5)P_2$. To explain this difference, we suggest that the nonconserved residues flanking site B (Figure 6; Raf-1 H79 vs A-Raf Y42, Raf-1 M83 vs A-Raf D46, and in particular Raf-1 P93 vs A-Raf Q56) could alter the orientation of these important basic residues within the structure and thus the lipid binding specificities of these two proteins. Structural information for the RBD of A-Raf itself, and a comparison with the structure of the RBD of Raf-1, is required to resolve the question of how phosphoinositide lipid binding specificity is determined.

The binding site for PA has previously been localized within the Raf-1 kinase domain (amino acids 389−423) to the sequence VLRKTRH (*8*). The corresponding region in A-Raf has an identical sequence and the two underlined basic residues within the A-Raf sequence correspond to site G, the mutation of which severely reduced binding to PA. Thus our data are consistent with A-Raf binding to PA through an analogous sequence to that used by Raf-1. Mutation of other BXB sequences within the A-Raf kinase domain, at sites F (TVFRGRW) and H (TVKTRW), also show reduced interactions with PA, suggesting that several regions may be involved in PA binding. Alternatively these additional regions may be important for maintaining the folded structure necessary for A-Raf to bind PA. Whether mutations of the corresponding regions of Raf-1 that have similar sequences (TVYKGKW and TVKSRW, respectively) will also reduce binding to PA is not known.

Previous reports have shown that Raf-1 can bind to and be regulated by PA, PS, and ceramide (*30*). These lipids have been suggested to assist in the plasma membrane association of Raf-1, in conjunction with the growth factor-dependent interaction of Raf-1 with activated Ras-GTP. Our results are consistent with Raf-1 binding to PA and PS (ceramide was not tested), and we also observe interactions with the monophosphoinositides PI(3)P, PI(4)P, and PI(5)P, as well as $PI(3,5)P_2$. Additional reports have shown that Raf-1 is associated with endocytic vesicles after insulin stimulation (*11*) and that Ras signaling may actually occur on endosomes as well as at the plasma membrane (*31*). This suggests that Raf-1 binding to phosphorylated PI lipids may play a significant role in the association of Raf-1 with specific membrane microdomains such as those on endosomes.

Mutation of A-Raf residues K50 and R52 within site B selectively prevented binding to $PI(4,5)P_2$ but not to other phosphoinositides [PI(3)P, PI(4)P, PI(5)P, and $PI(3,5)P_2$]. Our results suggest that a distinct region of the A-Raf protein is responsible for its binding to monophosphoinositides and $PI(3,5)P_2$. It has been suggested that the interaction of Raf-1 with both PA/PS and Ras-GTP increases the binding affinity of Raf-1 for the plasma membrane. It is also possible that the ability of a protein to bind to both a lipid and a protein at the same time (or two proteins, or two lipids) may act like a "ZIP code" to target that protein to a highly specific microlocation. The ability of a protein to bind two moieties at the same time, or in succession, could also allow a protein to be "handed off" to maintain tight control over its binding partners and location.

Analysis of the phenotypes of knockout mice for the different Raf family members (*32*−*36*) has led to the suggestion that the B-Raf family member is likely the critical Raf kinase responsible for MEK/MAPK pathway activation in most cell types (*6*). Further, Raf-1 and A-Raf appear to have critical cell survival functions distinct from their effects on the MEK/MAPK pathway (*1*). The kinase activities of

Raf-1, and particularly A-Raf, toward MEK have also been shown to be substantially lower (at least in vitro) than that of B-Raf (*37*−*39*). Raf-1 and A-Raf must be phosphorylated on several serine and tyrosine residues to attain full activity, suggesting that they are more highly regulated than B-Raf (*40*). The role of B-Raf as the key Raf kinase downstream of Ras is further supported by the discovery of frequent B-Raf mutations in human cancers (*41*) and the ability of these mutations to compromise B-Raf activity (*42*). Together, this has led to the suggestion that Raf-1 and A-Raf may have alternative functions, including additional substrates and downstream targets distinct from MEK (*2*, *4*−*6*, *43*). One potential Raf-1-specific target is Rb, since it has been shown that Raf-1 phosphorylates and inactivates Rb (*44*).

We have identified the p85 subunit of phosphatidylinositol 3′-kinase as an A-Raf-specific binding partner (*19*, *45*). During our characterization of the functions of the p85-A-Raf complex in cells, we have found that A-Raf can selectively regulate the ability of activated platelet-derived growth factor receptors to bind and activate phospholipase Cγ1 signaling pathways (*46*). In addition, this work also shows that A-Raf can regulate p85-associated phosphatidylinositol 3′-kinase activity, independent of its effects on the receptor. These are new functions for the A-Raf kinase.

Two additional examples of isoform-specific binding partners for A-Raf are the mitochondrial import proteins (hTOM and hTIM). This, coupled with the observation that A-Raf has been found in mitochondrial preparations, suggests that A-Raf may exert its cell survival functions by direct effects on mitochondrial proteins, possibly by phosphorylation (*43*). Perhaps different lipid binding specificities contribute to these isoform-specific Raf functions by regulating Raf association with critical target membranes.

In conclusion, we show that A-Raf and Raf-1 can bind to phosphoinositides, in addition to PA and PS, previously identified Raf-1 binding lipids. While both Raf kinases bind monophosphorylated phosphoinositides and PI(3,5)P_2, only A-Raf binds well to PI(4,5)P_2. We have identified two basic residues within the RBD of A-Raf that are required for PI(4,5)P_2 interaction, K50 and R52. The discovery that Raf kinases can bind specific phosphoinositides raises the possibility that these interactions could fine-tune their localization to specific membrane microdomains and that this could regulate their isoform-specific functions.

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