Functional Analysis of a Phosphatidic Acid Binding Domain in Human Raf-1 Kinase

MUTATIONS IN THE PHOSPHATIDATE BINDING DOMAIN LEAD TO TAIL AND TRUNK ABNORMALITIES IN DEVELOPING ZEBRAFISH EMBRYOS*

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Previously, we and others identified a 35-amino acid segment within human Raf-1 kinase that preferentially binds phosphatidic acid. The presence of phosphatidic acid was found to be necessary for the translocation of Raf-1 to the plasma membrane. We have now employed a combination of alanine-scanning and deletion mutagenesis to identify the critical amino acid residues in Raf-1 necessary for interaction with phosphatidic acid. Progressive mutations within a tetrapeptide motif (residues 398–401 of human Raf-1) reduced and finally eliminated binding of Raf-1 to phosphatidic acid. We then injected zebrafish embryos with RNA encoding wild-type Raf-1 kinase or a mutant version with triple alanine mutations in the tetrapeptide motif and followed the morphological fate of embryonic development. Embryos with mutant but not wild-type Raf-1 exhibited defects in posterior axis formation exemplified by bent trunk and tail structures. Molecular evidence for lack of signaling through mutated Raf-1 was obtained by aberrant in situ hybridization of the ntl (no tail) gene, which functions downstream of Raf-1. Our results demonstrate that a functional phosphatidate binding site is necessary for Raf-1 function in embryonic development.

The protooncogene Raf-1 kinase plays a crucial role in several normal and pathologic cellular processes including proliferation, differentiation, development, senescence, programmed cell death, cell cycle progression, immune responses, and carcinogenesis (1–3). Raf-1 functions downstream of p21 Ras (4, 5) and serves as an upstream regulator of the Ras-Raf-MEK-1-MAP kinase signal transduction cascade that is activated in response to a wide variety of signals, including growth factors, differentiation hormones, tumor promoters, inflammatory cytokines, calcium mobilization, DNA-damaging agents, and oxygen radicals. A common aspect of Raf-1 activation is its translocation to the plasma membrane, which is composed predominantly of acidic (−30%) and zwitterionic (70%) phospholipids (6, 7). Membrane lipids also function as second messengers for several intracellular signal transduction events. In the case of Raf-1, lipids such as ceramide and leukotriene D4 are related to its activation in select experimental systems (8, 9). Based on the requirement for membrane translocation of Raf-1 prior to activation, we investigated whether membrane phospholipids might be involved in a functional interaction with Raf-1 kinase that can be a precursor to subsequent activation. In vitro analysis of Raf-1-lipid interaction reveals two distinct phospholipid binding sites within Raf-1 kinase (site I and site II; see Refs. 10 and 11). Site I is located between amino acid residues 139 and 184 of human Raf-1 kinase and consists of a zinc-coordinating cysteine-rich domain analogous to domains present in protein kinase C and other proteins (reviewed in Ref. 13). Site I interacts with anionic phospholipids such as phosphatidylserine, predominantly via electrostatic interactions, driven by a cluster of basic amino acid residues (12). A second phospholipid binding site (site II) is located between residues 390 and 423 of human Raf-1. This region displays preferential interaction with phosphatidic acid, and the binding is not dependent on ionic interactions alone.

An increase in membrane phosphatidic acid via activation of phospholipase D by the tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (14) correlates with a net translocation of Raf-1 from the cytosol to the plasma membrane of Madin-Darby canine kidney cells (11). When the generation of phospholipase D-derived phosphatidate is inhibited by ethanol (via formation of phosphatidylethanol), a specific, dose-dependent loss of Raf-1 translocation is observed. These results suggest that agonist-induced Raf-1 translocation is coupled to the generation of phospholipase D-derived phosphatidic acid. Because translocation is a prerequisite for Raf-1 activation, we postulated that phosphatidic acid might regulate Raf-1 activation by enabling translocation. Subsequently Rizzo et al. (15, 16) demonstrated that in Rat-1 fibroblasts overexpressing the human insulin receptor (HIRB cells), the stimulation of the MAP kinase pathway by insulin is dependent on phospholipase D activation and is mediated via an induction of Raf-1 translocation to the plasma membrane and early endosomes by PA. The generation of PA is essential for Raf-1 translocation and brefeldin A, an inhibitor of the ADP-ribosylation factor (required for phospholipase D activation), prevents the translocation of Raf-1 in a dose-dependent manner. This inhibition of Raf-1 translocation can be reversed by exogenously added PA. Additionally, Raf-1 translocation in response to PLD-derived PA is also observed in Rat-1 cells expressing constitutively activated p21Ras (Q61L mutant) suggesting that PA and Ras may act concurrently and by mutually independent pathways to promote Raf-1 translocation to the plasma membrane (reviewed in Ref. 36). Based on the results from our experiments and that of...
Rizzo et al. (15, 16), we postulate that one of the mechanisms by which PA activates the MAP kinase cascade is via the induction of Raf-1 translocation to cellular membranes. However, whether the proposed PA-Raf-1 interaction does indeed play a functional role in the biology of an intact organism is currently unknown. The present study addresses this question by first identifying the molecular nature of PA-Raf-1 interaction and then determining the biological consequences of disrupting such interaction in a model system of vertebrate development.

EXPERIMENTAL PROCEDURES

Chemicals

96-well microtiter plates (Probind) were obtained from BD Biosciences. Glutathione-agarose matrix (sulfur-linked), phosphate-buffered saline, and bovine serum albumin (fraction V) were purchased from Sigma. All phospholipids tested were purchased from Avanti Polar Lipids. All other chemicals used were of the highest available commercial grade.

Pisciculture Reagents and Antibodies

Polyclonal anti-rabbit Raf-1 antibody (C12) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-GST rabbit polyclonal antiserum was a gift from Dr. Andrew Quest (University of Lauzanne, Lauzanne, Switzerland).

Methods

Expression and Purification of GST Fusion Proteins—All the GST fusion proteins used in this study were obtained from DNA generated by PCR using a FLAG-Raf-1 plasmid template (a gift from Dr. Roger Davis, University of Massachusetts, Worcester, MA). Details of the PCR, cloning, bacterial expression, and purification of the fusion proteins have been described previously (10). Site-directed mutagenesis on a phosphatidate binding fragment of Raf-1 kinase was performed via PCR as described (17). The PCR primers shown in Table I were used for the generation of the GST-Raf-1 fusion proteins in this study (alanine mutations are denoted by the code GCT). Following affinity purification of the expressed GST-Raf-1 fusion proteins over glutathione-agarose columns, the fusion proteins were analyzed for purity by SDS-PAGE and stored in aliquots at −80 °C. All fusion proteins were expressed in the Echerichia coli strain, BL-21, obtained from Novagen (Madison, WI).

Enzyme-linked Immunosorbent Assay Format Assay—The interaction of all GST-Raf-1 fusion proteins with different lipids was assessed by the enzyme-linked immunosorbent assay format essentially as described earlier (35).

Maintenance and Breeding of Zebrafish—Adult zebrafish were obtained from local pet stores and were maintained on a 14-h light/10-h dark cycle at 28.5 °C. For breeding, single male and female fish were placed in a breeding tank consisting of 1 gal plastic tank that was placed inside a 2.5-gallon tank. The bottom of the plastic tank had been removed and replaced with plastic mesh so that the eggs would pass through the mesh and would be collected at the bottom of the larger tank.

In Vitro Transcription and RNA Injection—Raf constructs were cloned in pGEM4z (gift of Paul Krieg, University of Arizona), a pGEM4z derivative that contains Xenopus β-globin 5′- and 3′-untranslated sequences and an A_{5′}A_{3′} sequence inserted downstream from a T7 polymerase promoter site. Constructs were linearized downstream from the A_{5′}A_{3′} sequence and were transcribed in vitro using a mMessage mMachine kit (Ambion), following the recommended protocol of the manufacturer. Following DNase treatment, phenol extraction, and ethanol precipitation, the RNAs were quantitated either spectrophotometrically or by RiboGreen (Molecular Probes, Inc.). RNAs were injected into embryos in 0.1× KCl, 0.2% phenol red at a concentration of 100 ng/μl to 1 μg/μl using an Eppendorf 5242 pressure injection apparatus and sterile Fomital.

RT-PCR Amplification of Zebrafish Phospholipase D1 cDNA Sequences—Degenerate primers 5′-GAYTGGTGGYTITCICCIGA-3′ and 5′-TACCTTTATGCCGTTGTTATTC-3′ were synthesized and probe 5′-ACTTCTCGGCTGACATTCTGCTTTTC-3′ were synthesized based on the sequence of the zebrafish phospholipase D cDNA sequence and were used for Taqman quantitative real time RT-PCR, using 25 ng of DNased total RNA from embryos at different stages of development.

RESULTS AND DISCUSSION

Identification of Key Residues That Mediate Raf-1-Phosphatidate Interaction—Employing deletion mutagenesis, we
previously identified a 35-amino acid segment within the carboxyl terminus of Raf-1 kinase (site II) that interacts with phosphatidic acid (11). Analysis of site II amino acid sequences of Raf-1 isoforms (A-Raf, B-Raf, Raf-1) and Raf-1 from different species reveals two subdomains of significant sequence homology. The first subdomain includes a cluster of basic amino acids corresponding to residues 398–401 of human Raf-1 mRNA (GenBank™ accession number X03484; Locuslink number 5894). The second subdomain contains predominantly hydrophobic residues encoded by amino acids 404–407. A Kyte-Doolittle hydrophilicity profile analysis of site II identified two distinct regions of hydrophilic and hydrophobic characters that matched the two sequence homology domains (data not shown).

We analyzed the relative contribution of each homology domain toward interaction of Raf-1 with phosphatidic acid via alanine-scanning mutagenesis of individual amino acids. A total of 18 different site II mutants were generated by PCR-based mutagenesis and expressed as GST fusion proteins in E. coli. Fig. 1a is a schematic depiction of all the mutants used in this study. We then tested the ability of the mutated GST-Raf-1 fusion proteins to interact with phosphatidic acid via an in vitro assay as described previously (10). The results are shown in Fig. 1, b–d. Fig. 1b shows the binding curves generated with proteins that contain mutations within the basic residues of the first homology domain of site II. Single site R398A or R401A replacements did not affect phosphatidate binding when compared with the wild-type protein. K399A replacement resulted in reduced binding to phosphatidic acid. The two-site replacement mutants (R398A, R401A) also displayed reduced binding to PA compared with the wild-type protein. The reduction in binding was more severe in the two-site mutants containing mutations in Lys-399 (R398A, K399A, and K399A, R401A, respectively). Finally, the binding was severely compromised (80–95% reduction compared with wild-type) in a triple site mutant (R398A, K399A, R401A). These results suggested that the basic cluster, RKTR, constitutes a critical determinant for Raf-1-PA interaction with Lys-399 probably providing the major contribution.

We then focused on the second region of homology within site II, encompassing the hydrophobic residues, and created single site alanine mutants for residues 402–410 of human Raf-1. The mutated proteins were again tested for their relative ability to bind phosphatidic acid by the enzyme-linked immunosorbent assay format assay described previously. The results, shown in Fig. 1c, demonstrate that site-directed mutagenesis within this domain had only a modest effect on interaction with phosphatidic acid. Replacement of Val-403, Leu-406, Leu-407, and Phe-408 to alanine did not change the binding profile to phosphatidic acid, compared with the wild-type sequence. Mutations of His-402, Ile-405, and Met-409 resulted in slightly reduced binding to phosphatidic acid. The greatest reduction in phosphatidate binding (about 40% of control) was observed with the Asn-404 and Gly-410 mutants. A comparison of the effects of mutations within the first and second homology domains of site II suggests that the charged tetrad sequence, RKTR, probably

binding site. b, relative PA binding data with alanine-scanning mutations within residues 398–401 of human Raf-1 kinase at various concentrations of the mutant proteins. Mutant proteins were used as GST fusions in the binding assay as described in the text. c, binding results for a second set of Raf-1 mutants encompassing predominantly hydrophobic residues 402–410 of human Raf-1. d, a comparison of relative binding affinities for Raf-1 mutants used in b and c at a fixed concentration of 100 nM GST fusion protein. Data shown in b and c are from a representative binding study performed in duplicate for each protein concentration. d displays average binding values from multiple experiments, where the average binding to wild-type Raf-1 is normalized to 1.
constitutes a major determinant for binding to phosphatidate and may provide an initial, electrostatic-driven clustering around one or more phosphatidate head groups. The hydrophobic region, adjacent to RKTR, may subsequently function to associate with phosphatidic acid via non-ionic interactions, resulting in a more stable complex. However, in the absence of the primary interaction driven by the charged amino acids, the hydrophobic residues are not sufficient to form stable association with phosphatidate. Additional support in favor of the critical role of the RKTR sequence in binding to phosphatidate is provided by a site II mutant lacking the tetrapeptide motif altogether (RafDel398–401). As expected, the corresponding mutant protein failed to interact with PA. A comparison of relative PA binding by all the site II mutants employed in this study is shown in Fig. 1.

To examine whether the RafAAA mutation would affect normal development, synthetic RNAs encoding wild-type human Raf, RafK375M, or RafAAA were injected into one-two cell zebrafish embryos. Previous work in Xenopus has shown that injecting the RafK375M allele into one-two cell embryos results in embryos lacking trunk and tail structures (26). The RafK375M mutation replaces a critical lysine residue at the ATP binding pocket of Raf-1 resulting in an inactive protein. As shown in Fig. 2 injection of wild-type Raf had no effect on normal zebrafish development, whereas about 30% of embryos injected with either RafAAA or RafK375M produced a phenotype similar to that seen in Xenopus; in 24 hours post-fertilization (hpf) embryos the tail is absent or severely shortened, and the overall length of the anterior-posterior axis is shortened. Examination of injected embryos at 12 hpf frequently revealed that the notochord was bent or shortened (data not shown). Measurement of the steady state levels of human Raf-1 in injected embryos by immunoblotting showed comparable protein levels in embryos injected with the different forms of Raf-1 (data not shown).

One of the known downstream targets of FGF signaling during vertebrate gastrulation is the product of the Xbra or ntl gene. Expression of a dominant negative FGF receptor or a mutant allele of one of several different MAP kinase cascade components results in a reduction or elimination of normal Xbra expression. Conversely, overexpression of FGF or a constitutively active MEK or treatment of Xenopus animal caps with FGF causes an increase in Xbra expression (20). To assess the effects of injecting the various forms of Raf-1 on ntl expression, whole mount in situ hybridization was performed using a digoxigenin-labeled sense and antisense probe to ntl. ntl expression normally begins at 5.5 hpf. At this time (germ-ring stage) the germ ring forms and is considered the start of gastrulation. In normal embryos, ntl expression is restricted at the germ-ring stage to a circumferential belt of cells that have newly invovled from the epiblast layer inwards toward the yolk, forming the mesendodermal cell layer (Fig. 3a). In embryos injected with either RafAAA or RafK375M but not wild-type Raf-1 this belt of expression is disrupted, resulting in embryos with a discontinuous band of cells expressing ntl (Fig. 3b). Thus, injection of Raf-1 with a mutated phosphatidate binding site is sufficient to block normal signaling events that are required for the expression of ntl in the newly formed mesendodermal layer.

Phosphatidic acid can be generated by two different mechanisms. In one mechanism, glycerol is phosphorylated by enzymes belonging to the diacylglycerol kinase family to yield PA. The other mechanism involves the hydrolysis of phospholipids such as phosphatidylcholine to PA by the action of the phospholipase D group of enzymes. The relative contribution of these reactions toward the generation of PA in zebrafish em-
Bryo expression is restricted to specific cell types or the 12 somite stage. The results obtained from the Taqman were normalized to beta-actin RNA levels in the same sample. "MBT" refers to the mid-blastula transition.

**Fig. 5. Detection of a zebrafish phospholipase D transcript in embryos by whole mount in situ hybridization.** Hybridization probe was generated from a partial clone of zebrafish PLD. a, 64-cell stage; b, shield stage; c, 12 hpf. Embryos hybridized with a sense strand phospholipase D probe were negative (data not shown).

**Fig. 4. Expression analysis of zebrafish PLD by Taqman.** mRNA Stage refers to the developmental stages of the zebrafish embryo from where the RNA was obtained; AvgCt refers the average detection threshold (Ct) observed from duplicate assays; 40-AvgCt is the value obtained by subtracting AvgCt from a Ct of 40 (a Ct of 40 implies no expression of RNA in that sample); Relative abundance is calculated by raising 2 to the power of 40-AvgCt and gives an estimate of relative enrichment compared with no expression; Fold over 2-4 cells compares the relative abundances of zebrafish PLD message determined in all RNA samples to that observed for the two-four cells sample. All values obtained from Taqman were normalized to beta-actin RNA levels in the same sample. "MBT" refers to the mid-blastula transition.

**Fig. 3. Detection of ntl gene expression in 5.5-hpf zebrafish embryos by whole mount in situ hybridization.** In embryos injected with wild-type Raf-1 (bottom), ntl expression is restricted to a continuous circumferential belt of cells. In embryos injected with RafAAA (top), this belt of expression is disrupted leading to a discontinuity in ntl expression.
specific regions of these embryos. These results indicate that phospholipase D transcripts are present before the onset of zygotic transcription at the mid-blastula transition and are present in both ectodermal and mesendodermal cells at later stages.

CONCLUSIONS

We previously identified a segment within human Raf-1 kinase (amino acid residues 390–423) that binds PA. We also showed that PA is required for the translocation of Raf-1 from the cytosol to the plasma membrane in Madin-Darby canine kidney cells stimulated with the phorbol ester, 12-o-tetradecanoyl phorbol-13-acetate. Rizzo et al. (15) demonstrated the requirement for PA in the translocation of a GFP-Raf-1 fusion protein in response to insulin stimulation. In their studies, PA did not directly activate Raf-1 in vitro or in vivo strongly arguing for a role of PA primarily in facilitating the translocation of Raf-1 kinase.

The involvement of PA in binding Raf-1 and mediating its agonist-dependent translocation is consistent with the effect of lipid second messengers on the MAP kinase pathway. Many of the signals that activate the MAP kinase pathway also activate phospholipase D (22). Additionally, phospholipase D is also regulated by heterotrimeric G proteins, G13 and G12 (23). This is suggestive of a role of PA as a lipid second messenger (24, 25).

The data generated from our group and that of Rizzo et al. (15) identifies a molecular mechanism by which PA might be exerting its regulatory function.

In the present work, we have identified, via alanine-scanning mutagenesis, individual amino acid residues within Raf-1 that are critical for its interaction with PA. In vitro binding assays, employing GST-Raf-1 fusion proteins (containing either the wild-type PA binding fragment or mutated versions thereof), have identified a tetrad of charged residues (RKTR, residues 398–401) required for PA binding. Mutation at the lysine residue (Lys-399) in the tetrad significantly inhibits PA binding. Conversion of all three charged residues to alanine, or their deletion, leads to total loss of binding. We conclude that the RKTR tetrad is the major contributor toward the interaction of site II within Raf-1 with PA. In this context it is worthwhile to compare the PA binding site mutations employed by Rizzo et al. (16) to those used in the current study. Rizzo et al. (16) observed that conversion of the first arginine of the RKTR tetrad into alanine (R398A) in full-length Raf-1 was sufficient to block the translocation of the mutant protein to endosomes in response to insulin. They also expressed a GFP fusion protein linked to a 36-amino acid sequence from Raf-1 containing the PA binding site (GFP-PABR, equivalent to site II in this study). The native GFP-PABR blocked Raf-1 translocation to endosomes (by sequestration of PA) and also blocked MAP kinase activation. However, GFP-PABR containing a single mutation equivalent to R398A was still capable of interacting with PA and prevented the translocation of native Raf-1. This result is unexpected in light of the behavior of full-length Raf-1 harboring the R398A mutation. It is only when a second mutation was introduced (equivalent to R401A), that the GFP-PABR lost its ability to prevent Raf-1 translocation and MAP kinase activation. In our hands, a single mutation at R398A in a GST-site II fusion protein does not significantly reduce binding to PA. We find instead that the point mutation K399A produces a dramatic reduction in PA binding, and any dual mutations in the RKTR tetrad that include K399A cause significant loss of binding.

Lu et al. (27) described a study involving the isolation of temperature-sensitive mutations in the catalytic domain of Raf-1 and the expression of conditionally active and dominant-defective forms of Raf-1 in cultured mammalian cells. The authors introduced pairwise alanine-scanning mutations into the entire ATP binding subdomain of a mutant Raf-1 that was truncated for the first 334 amino-terminal residues and displayed a kinase activity comparable with that of oncogenic v-raf (28). Two of the alanine-scanning mutations were for residues R398A,K399A and K399A,R401A, the same residues identified for PA binding. Upon electroporation into the rat fibroblast cell line, TGR-1, each of the mutant proteins exhibited a behavior similar to wild-type Raf-1 as measured by two independent biological assays for v-Raf function (focus formation and growth in soft agar). This suggests that Arg-398, Lys-399, and Arg-401 are not involved in ATP binding, and, significantly, mutations at those sites do not interfere with the catalytic activity of Raf-1. These results further suggest that unlike full-length Raf-1, where loss of PA binding leads to a dominant negative effect, the constitutively activated, NH2-truncated version of Raf-1 is not subject to regulation by PA (or p21Ras) and consequently does not display a dominant negative phenotype when the PA binding sites are mutated.

The RKTR tetrad is positioned within the ATP binding domain of Raf-1 (343–426). X-ray crystallographic studies in other kinases such as protein kinase A, Cdk2, MAP kinase, and twitchin kinase (29–32) have indicated that the ATP binding domain is a small, compact, and conserved structure and is largely independent of other structural entities within the kinase such as the substrate binding domain and other regulatory domains. Therefore, it is unlikely that mutations in Arg-398, Lys-399, and Arg-401 would affect the conformation of the MEK binding and p21Ras binding domains of Raf-1, which are situated in the upstream amino-terminal portion of the protein.

This study elucidates the amino acid residues primarily responsible for mediating an interaction between site II of Raf-1 kinase and PA. The functional consequences of mutations at these amino acid residues have been followed in developing zebrafish embryos. Our results indicate that in the presence of Raf-1 kinase deficient in binding PA via site II, the developmental program in zebrafish embryos is severely compromised, leading to embryos with bent tail/trunk structures and a shortened anterior-posterior axis. Our work is consistent with previous work in a number of animal model systems that have shown that Raf is required for normal embryonic development. Thus, a mutation in the ATP binding pocket of Raf (RafK375M) acts as a dominant negative in Xenopus embryos, resulting in embryos with shortened body axes and defects in the processes by which mesoderm is formed and specified. Similarly, mice lacking A-Raf, B-Raf, or c-Raf-1 exhibit a number of defects in development and organogenesis (33). Drosophila D-Raf acts downstream of a number of receptor tyrosine kinases that are required for normal development, including the EGF, FGF, torso, and sevenless receptors. Similar work with other components of the MAP kinase cascade indicates a role for this pathway in the normal specification of the anterior posterior and dorsal ventral axes. Studies indicate that similar pathways control germ layer formation and axis specification in zebrafish (34). Thus, the normal processes by which mesoderm is formed and the anterior posterior axis is specified provides a readout for perturbations in the normal signaling events, including the overexpression of dominant negative forms of c-Raf-1 kinase. Our results indicate that in the presence of Raf-1 kinase deficient in binding PA via site II, the development program in zebrafish embryos is severely compromised, leading to embryos with bent tail/trunk structures and shortened anterior-posterior axis. This phenotype effect of mutated Raf-1 is corroborated at the molecular level by a concomitant disruption in the expression of the ntl gene, signifying impaired FGF signaling.
In addition to the results reported here, Raf-1:PA Binding and Embryonic Development

There are several possible explanations for this dominant negative effect. It is possible that the non-physiological levels of externally introduced RafAAA in the embryos may result in its direct interaction with its substrate, MEK, thereby making MEK unavailable for the endogenous Raf proteins. However, lack of an intact PA binding site does not allow the externally introduced RafAAA to be appropriately translocated and subsequently activated by Ras resulting in a net dominant negative effect. Alternatively, as reported previously (40), Raf-1 proteins are known to form homodimers. It is conceivable that RafAAA will oligomerize with endogenous Raf-1. However, because of its inability to bind PA, the RafAAA-endogenous Raf complex may not be effectively translocated to the membrane thereby preventing the activation of the endogenous Raf. Finally, it should be noted that a recent study (37) investigated Raf-1:PA binding site inhibiting the proper membrane attachment of Raf-1, thus resulting in a net dominant negative effect. Consequently, activated, farnesylated Ras was minimal, suggesting that the recruitment and binding by GTP-Ras primarily via diffusion in the plane of the membrane-prebound Raf-1 would involve Raf recruitment and binding to GTP-Ras primarily via diffusion in the plane of the membrane such that Raf-1 is structurally and positionally reconfigured for interaction with activating kinases and substrates. If such lipids are indeed present at that stage in the embryos, the functional PA binding site inhibits the proper membrane attachment of RafAAA required for its activation. Consequently, a dominant negative phenotype is observed. Whatever the mechanism by which RafAAA exerts its dominant negative effect, these results strongly implicate that the regulation of Raf-1 kinase by PA is an essential component of normal Raf-1 function in biological systems.

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