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PHOSPHOLIPASE A_2 AND LIPIDS AS POTENTIAL MODULATORS OF c-Raf-1 KINASE

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c-Raf-1 is a proximal serine/threonine kinase in the signaling cascade of many mitogens. The cellular mechanisms responsible for regulation of this kinase remain ill-defined. Although c-Raf-1–associated proteins have been identified, including Ras, none of these have been found to activate c-Raf-1 kinase *in vitro*.

To evaluate whether arachidonic acid or one of its products is implicated in c-Raf-1 activation, c-Raf-1 activity was measured in LLC-PK_1 kidney epithelial cells overexpressing the 100 kDa phospholipase A_2 (PLA_2). As compared to control neomycin plasmid transfected cells, the cells overexpressing PLA_2 had a greater activation of c-Raf-1 in response to A23187 and phorbol ester stimulation.

To explore the possibility that c-Raf-1 activity may be modulated directly by lipids, the enzymatic characteristics of c-Raf-1 were determined, and the effects of various possible lipid modulators on c-Raf-1 activity were examined. The K_m of c-Raf-1 for ATP and mitogen-activated protein kinase kinase (MAPKK), the only known physiologic substrate of c-Raf-1, were 11.6 μM and 0.8 μM, respectively. Of 13 lipids or combinations of lipids tested, including arachidonic acid and several eicosanoids, only phosphatidylserine and diacylglycerol in the presence of Ca^{2+} (2.5 mM) increased c-Raf-1 kinase activity significantly. The increase (1.5-fold) was approximately two orders of magnitude less than the stimulation of protein kinase C by these lipids.

c-Raf-1 kinase activity and immunoreactivity eluted on gel filtration at a predicted molecular mass of greater than 150 kDa, suggesting that active c-Raf-1 is part of a multimeric complex. The absence of immunoreactive Ras in the active fractions confirms that the interaction is not necessary to maintain c-Raf-1 in an active state.

In conclusion, a product of PLA_2 may play a role, together with Ras and another unidentified cofactor, in activating c-Raf-1. This lipid mediator(s) may directly or indirectly regulate the activity of c-Raf-1, but the identity of the mediator and its mode of interaction with c-Raf-1 and its associated proteins remain unclear.

Keywords: lipids, eicosanoids, mitogen-activated protein kinases, LLC-PK_1 cells.

INTRODUCTION

c-Raf-1 is the cellular homologue of v-Raf, the transforming gene of mouse sarcoma virus, 3611 [1]. It encodes a serine/threonine kinase that is activated by many mitogens, including G-protein–linked agonists endothelin, thrombin [2], growth factors (platelet-derived growth factor [PDGF], epidermal growth factor [EGF], colony-stimulating factor-1 [CSF-1], and fibroblast growth factor [FGF]), cytokines, and phorbol esters (reviewed in Refs. 3 and 4]. The importance of c-Raf-1 in the control of proliferation is supported by the finding that c-Raf-1 functions downstream of Ras proteins in the signaling cascade and is essential for growth induction by serum factors and protein kinase C [5]. c-Raf-1 is the most proximal kinase identified in a mitogen- and oncogene-stimulated protein kinase cascade that includes mitogen-activated protein kinase kinase (MAPKK) and MAP kinases. Oncogenic vari-

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ants of c-Raf-1 and mitogen-stimulated wild-type c-Raf-1 can phosphorylate and activate MAPKK [2, 6–8], which in turn activates MAP kinase [9–11]. The MAP kinases then phosphorylate and activate a number of substrates, including ribosomal S6 kinase, ($p85^{rsk}$), $cPLA_2$, and the transcription factors, Elk-1/$p62^{TCF}$, ATF-2, and c-Jun [12–18].

The mechanism of activation of c-Raf-1 is not clear. Using biochemical techniques and the yeast two-hybrid cloning system, c-Raf-1 has been found to associate with Ras [19–23]. To date, however, this association has not been found to result in activation of c-Raf-1 *in vitro*. It appears that the major role of Ras interaction with c-Raf-1 is to bring the latter to the membrane where other as yet unidentified factors can activate the kinase [24, 25]. Ras interacts with c-Raf-1 via the latter's amino-terminal regulatory domain, which contains a cysteine finger region. Analogous to protein kinase C, this region is also predicted to be a lipid-binding region [26], suggesting c-Raf-1 activity may be modulated by lipid binding [4].

Because of its importance in the cascade of mitogen-activated kinases, it is important to understand the regulation of this enzyme. Previous data have supported the role of arachidonic acid and one or more of its products as important modulators of cellular proliferation [27, 28]. If PLA_2, arachidonic acid, or one or more of the eicosanoid metabolites of arachidonic acid, were activators of c-Raf-1, then one might predict that overexpression of PLA_2 would enhance c-Raf-1 activity. We evaluated the activation of c-Raf-1 in response to a Ca^{2+} ionophore, A23187, and the phorbol ester, PMA, in an LLC-PK_1 renal epithelial cell line, which overexpresses PLA_2, as compared to cells transfected with the neomycin selection plasmid only. Our findings support the hypothesis that arachidonic acid and/or one or more of its metabolites are important intermediates in the cellular signal transduction pathway used by peptide hormones and growth factors to induce a proliferative response, and suggest part of this effect may be via modulation of c-Raf-1 activity. A number of eicosanoids and other lipids were tested for direct activation of c-Raf-1 *in vitro;* however, the identity of a lipid mediator responsible for activation of c-Raf-1 remains unknown. Gel filtration chromatography reveals that active c-Raf-1 exists as a multimeric complex, and activity is maintained in the absence of associated Ras.

METHODS

Materials

[γ-^{32}P]ATP was from DuPont–New England Nuclear. ATP (special quality) was from Boehringer Mannheim. *N*-ethyl maleimide, arachidonic acid, diacylglycerol (β-arachidonoyl γ-stearoyl), 1-oleoyl 2-acetyl *sn*-glycerol, phosphatidic acid (β-arachidonoyl γ-stearoyl), phosphatidylinositol bisphosphate, and phosphatidylserine were from Sigma. $PGF_{2\alpha}$, PGE_2, 12(S)-HETE and 14(15)-EpETrE acid were from Cayman Chemical. Geneticin 418 was obtained from Gibco BRL.

Cell culture

LLC-PK_1 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and supplemented with 1% L-glutamine. Stable cell lines transfected with pcDNA I/Neo or pMT2-$cPLA_2$ (obtained from Genetics Institute) were maintained in the same medium with the addition of G418 sulfate 400 μg/ml. The $cPLA_2$-transfected cells were designated LMPC2. Cells transfected with pcDNA I/Neo alone were designated LNeoC4.

Phospholipase A_2 activity

PLA_2 activity in 100,000 × g supernatants of LMPC2 cells was 15 times that of the LNeoC4 cells. PLA_2 activity was measured using 1-stearoyl-2-[1-^{14}C] arachidonoyl-*sn*-glycero-3-phosphocholine (2-[1-^{14}C]AA-GPC; 55.6 mCi/mmol^{-1}) purchased from Amersham, Buckinghamshire (UK), using standard techniques established in our laboratory [29].

Expression and purification of histidine-tagged c-Raf-1

For studies of candidate lipid modulators, c-Raf-1 was purified from SF9 insect cells infected with recombinant baculovirus, encoding a histidine-tagged c-Raf-1 either alone (producing "inactive" c-Raf-1), or together with recombinant baculovirus, encoding v-src and Val-12 v-ras (all obtained from Dr. Ulf Rapp) [30]. After 72 h of infection, the cells were lysed and the histidine-tagged protein was purified, using a Ni^{2+}-nitrilotriacetic acid-Sepharose column (Invitrogen) and imidazole elution [31]. c-Raf-1 was purified to a specific activity of 29,000 U/mg (where 1 U = 1 pmol of phosphate transferred to MAPKK per minute). The protein was dialyzed into a buffer containing 20 mM HEPES, pH = 7.4; 5 mM EGTA, 0.1% Triton X-100, 2 mM dithiothreitol (DTT), 50% glycerol, and 1 mM phenylmethylsulfanyl fluoride, and stored at −20°C.

Immunoprecipitation and assay of c-Raf-1 kinase

c-Raf-1 was immunoprecipitated from LLC-PK_1 cells using SP63 antisera (courtesy of Dr. Ulf Rapp), using methods previously described [2, 6]. Activity of the c-Raf-1 kinase, either purified from SF9 cells or in

immunoprecipitates, was measured by determining the phosphorylation of MAPKK purified from bovine brain. c-Raf-1 kinase activity was assayed in the presence of $MgCl_2$ (10 mM), [γ-^{32}P]ATP (100 μM, 3000–8000 cpm pmole^{-1}), and MAPKK (23 μg/ml). c-Raf-1 was assayed, using 5.6 ng per assay for 20 min. Reactions were stopped with SDS sample buffer, and the proteins were resolved by SDS-PAGE. Bands corresponding to the 48-kDa MAPKK were excised, and radioactivity was measured by liquid scintillation counting. All assays were corrected for MAPKK autophosphorylation, which was less than 10% of c-Raf-1 kinase activity. In assays examining potential lipid modulators of c-Raf-1, lipids were sonicated on ice in kinase assay buffer (50 mM β glycerol phosphate [pH = 7.3], 1.5 mM EGTA, 1 mM DTT, 0.03% Brij 35) for 1 min prior to being added to the reaction mix.

Purification of MAP kinase kinase

Bovine brain MAPKK was prepared as previously described [2, 6]. This procedure routinely yielded a preparation with a specific activity of 3500 U/mg with an overall recovery of 6%. The purity of the preparation was approximately 30% [32].

Gel filtration chromatography

Purified histidine-tagged active c-Raf-1 (200 μl) was applied to a 1 × 30-cm Superose 12 column (Pharmacia), equilibrated with 20 mM Tris, pH 7.4, 2 mM EGTA, 200 mM NaCl, 1 mM DTT, 0.05% Triton X-100, and 0.5-ml fractions, were collected. The column was calibrated with ferritin, 440 kDa; aldolase, 158 kDa or IgG, 150 kDa; bovine serum albumin, 67 kDa; and ovalbumin, 43 kDa or ribonuclease A, 14 kDa. Xylene cyanol was used to indicate included volume.

Immunoblotting

Samples were run on 10% SDS-PAGE and then transferred to Imobilon. After blocking, the membranes were exposed to affinity-purified anti-Raf antisera (anti-SP63), anti-Ras antisera (Oncogene Science), or a monoclonal anti-GTPase activating protein (GAP) antibody (Upstate Biotechnology Inc.). Antibody-binding was detected with the ECL Western blotting system (Amersham).

RESULTS AND DISCUSSION

Overexpression of PLA_2 enhances activation of c-Raf-1

The 100,000 ×g supernatants of LMPC2 cells contained 15-fold higher levels of PLA_2 activity when compared to LNeoC4 cells. c-Raf-1 activation in response to A23187 (1 μM) and PMA (100 nM) in LMPC2 cells was approximately twice that in LNeoC4 cells (Fig. 1a). This greater increase in c-Raf-1 kinase activity was found despite less immunoreactive c-Raf-1 in the cells overexpressing $cPLA_2$ (Fig. 1b).

Direct modulation of c-Raf-1 by lipids

We determined whether arachidonic acid, several eicosanoids, or other membrane lipids directly activated c-Raf-1 expressed in, and purified from, SF9 cells. To do this, we first characterized the kinetics of c-Raf-1 [32]. The Michaelis Boston K_m of c-Raf-1 for ATP and MAPKK were 11.6 μM and 0.8 μM, respectively. Activity of c-Raf-1 increased in a linear fashion when Mg^{2+} or Mn^{2+} concentrations were increased from 0 to 5 mM. Mg^{2+} was the preferred cation at concentrations of Mg^{2+} and Mn^{2+} above 5 mM [32].

There are many examples of regulation of intracellular signaling events by lipids, including arachidonic acid and its metabolites. Arachidonic acid alters ligand–receptor interactions [33, 34]. Arachidonic acid as well as other fatty acids, which are not substrates for cyclooxygenase or lipoxygenase enzymes, modulate activity of a number of different membrane channels [35–41]. Arachidonic acid increases the activity of guanylate cyclase [42], protein kinase C [43, 44], and a GTPase inhibitory protein [45]. It inhibits Ca^{2+}/calmodulin-dependent protein kinase II [46] and the GTPase activating protein, GAP [47]. Arachidonic acid also activates the nontransmembrane protein tyrosine phosphatase, containing two src homology 2 (SH2) domains (PTP1C, SHPTP-2, or Syp) [48]. This phosphatase, like protein kinase C, is also activated by anionic phospholipids. We have reported that endogenous products of arachidonic acid are important modulators of intracellular calcium homeostasis [49] and proliferation in rat mesangial cells [27].

Given this background of involvement of lipids in the regulation of intracellular enzymes important for signaling, together with the suggestion of a lipid-binding domain in the regulatory region of c-Raf, and our findings of enhanced activation of c-Raf-1 in cells overexpressing $cPLA_2$, we tested a number of lipids for potential modulatory effects on c-Raf. Of 13 lipids or combinations of lipids tested (Table 1), only phosphatidylserine and diacylglycerol, in the presence of 2.5 mM Ca^{2+}, increased c-Raf-1 kinase activity significantly, and the increase was only 1.5-fold [32]. This small increase was approximately two orders of magnitude less than the stimulation of protein kinase C by these lipids. Arachidonic acid, PGE_2, $PGF_{2\alpha}$, 12(S)-HETE, 14,(15)-EpETrE (Table 1), and lysophosphatidylcholine and lysophosphatidic acid (data not shown) had no effect. In addition, although phosphatidylcholine-

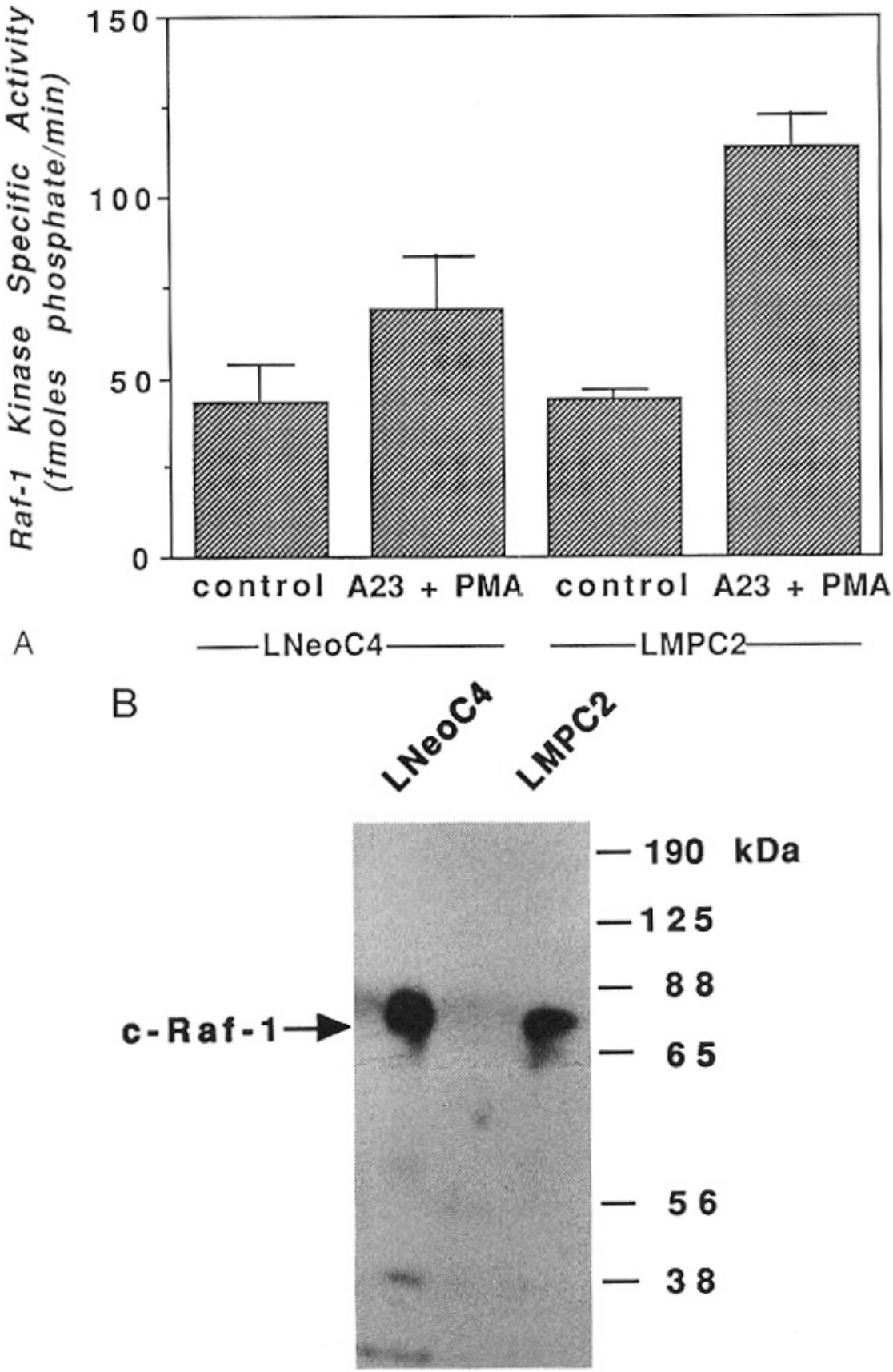

Fig. 1. Activation of c-Raf-1 by phorbol ester and A23187 in $cPLA_2$ overexpressing cells. LMPC2 cells, stably overexpressing $cPLA_2$, or LNeoC4 cells, transfected with the neomycin resistance plasmid (pcDNAI/Neo) only, were stimulated with PMA (100 nM) and A23187 (1 mM) for 10 min. Cell lysates were subjected to immunoprecipitation with SP63 antisera and aliquots were taken for c-Raf-1 kinase assay (a), or immunoblotting for c-Raf-1 (b).

Table 1. Candidate lipid modifiers.

Lipid	"Active" Raf (% C)	"Inactive" Raf (% C)
AA (150 μM)	98	110
DAG (100 μg/ml)	91	102
PA (100 μg/ml)	85	113
PIP_2 (100 μg/ml)	99	99
$PGF_{2\alpha}$ (150 μM)	112	129
PGE_2 (150 μM)	111	118
12(*S*)-HETE (150 μM)	101	105
14,(15)-EpETrE (150 μM)	110	105
Ca^{2+} (5 mM)	80	111
Phosphatidylserine (100 μg/ml)	84	113
Ca^{2+}/PS/DAG (2.5 mM/ 100 μg/m/25 μg/ml)	72	151
Ca^{2+}/PS/OAG (2.5 mM/ 100 μg/m/10 μg/ml)	74	158
n-Acetyl *s*-farnesyl cysteine (100 μg/ml)	74	157

Note: c-Raf-1 was preincubated with the sonicated lipids for 10 min at 30°C before performing the kinase assays. PIP_2, phosphatidylinositol biphosphate; 12(*S*)-HETE, 12-hydroxy[*S*,(*E*,*Z*,*Z*,*Z*)]-5,8,10,14-eicosatetroenoic acid; 14,(15)-EpETrE, 14,(15)-epoxy-eicosatrienoic acid; DAG, diacylglycerol; PS, phosphatidylserine; PA, phosphatidic acid. DAG and PA were β-arachidonoyl γ-stearoyl. (From Ref. 32, with permission.)

specific phospholipase C has been implicated in the activation of c-Raf-1 [50], the products of this enzyme, diacylglycerol (DAG) and phosphatidic acid, were also ineffective (Table 1). Thus, lipids alone were incapable of activating c-Raf-1 *in vitro*.

It might be argued that only a limited number of representatives of classes of potential lipid modulators were tested and that any c-Raf-1/lipid interaction could be very specific to a modulator not included in these studies. However, lipid activation of kinases or phosphatases has generally been found to be an effect common to a class or group of lipids [48, 51]. These data suggest that while a product of PLA_2 activation may play a role in activating c-Raf-1, either the lipids tested were not the correct ones or a critical cofactor necessary for activation was missing, or the effect we observed is indirect (possibly via modulation of a GAP or a GTPase inhibitory protein [45, 47]).

Gel-filtration chromatography of c-Raf-1

On gel-filtration chromatography, the peak of c-Raf-1 kinase activity and immunoreactivity eluted at a predicted molecular mass of greater than 150 kDa, suggesting that active c-Raf-1 exists as a multimeric complex (Fig. 2) [32]. Ras is a candidate protein that might be complexed to c-Raf-1 since Ras has been implicated in the localization of c-Raf-1 to the membrane via a direct protein–protein interaction [24, 25]. In agreement with the absence of Ras in immunoprecipitates of active c-Raf-1 [24], immunoreactive Ras was not found in fractions with active c-Raf-1 (data not shown). Thus, whereas Ras is necessary to bring c-Raf-1 to the membrane, Ras is not necessary to maintain c-Raf-1 in an active state.

CONCLUSIONS

The mechanism of activation of c-Raf-1, a proximal kinase in the signal transduction cascade leading to mitogenesis, remains unclear. Although Ras is essential to bring the kinase to the membrane, the factors present in the membrane which then activate c-Raf-1 are unknown. c-Raf-1 associated proteins have been identified, including hsp 90 and p50 [52], but none of these have been demonstrated to activate the kinase *in vitro*. Our data suggest that a product of PLA_2 activation may play a role in the activation of c-Raf-1, but our

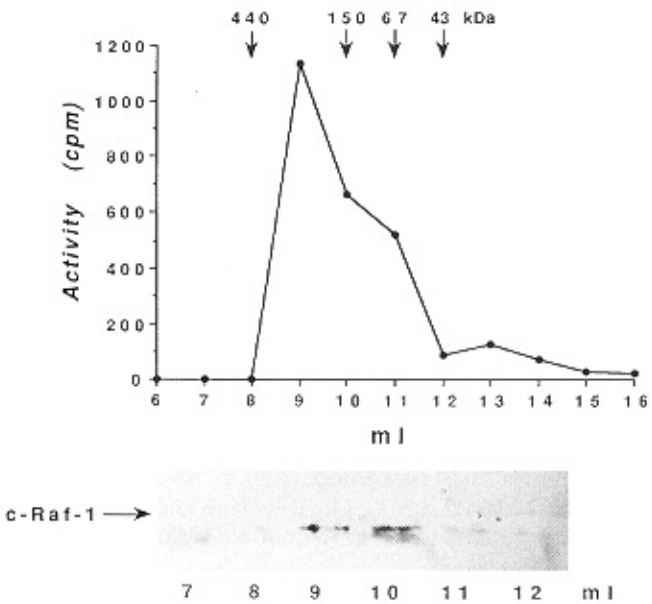

Fig. 2. Analytical gel filtration of histidine-tagged c-Raf-1 from triply-infected SF9 cells. Histidine-tagged c-Raf-1 was purified from SF9 cells triply-infected with baculoviruses encoding histidine-tagged c-Raf-1, v-src, and v-Ras, and subjected to gel filtration on a Superose 12 column. The column was developed at 0.5 ml/min and sixty 0.5-ml fractions were collected. Kinase assays were performed on every other fraction. Kinase activity of the fractions is presented as cpm of $^{32}PO_4$ incorporated into MAPKK. The elution position of standard proteins is indicated: ferritin (440 kDa); IgG (150 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa). Below the graph is an immunoblot of the column fractions using affinity-purified SP63 to identify c-Raf-1. (From Ref. 32, with permission.)

failure to activate the kinase *in vitro*, with the panel of lipids we tested, suggests that a critical cofactor may be missing or the effect is indirect. If lipid mediators regulate the activity of this kinase, the identification of the mediator and its mode of interaction with c-Raf-1 and its associated proteins, including Ras, will be a major step toward understanding how arachidonic acid and its metabolites modulate the mitogenic response.

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REFERENCES

1. Rapp UR, Goldsborough MD, Mark GE, Bonner TI, Groffen J, Reynolds FH JR, Stephenson JR: Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. Proc Natl Acad Sci USA 1983;80: 4218–4222.
2. Kyriakis JM, Force TL, Rapp UR, Bonventre JV, Avruch J: Mitogen regulation of c-Raf-1 protein kinase activity toward mitogen-activated protein kinase kinase. J Biol Chem 1993;268:16009–16019.
3. Rapp UR: Role of Raf-1 serine/threonine protein kinase in growth factor signal transduction. Oncogene 1991;6: 495–500.
4. Li P, Wood K, Mamon H, Haser W, Roberts T: Raf-1: A Kinase currently without a cause but not lacking in effects. Cell 1991;64:479–482.
5. Kolch W, Heidecker G, Lloyd P, Rapp UR: Raf-1 protein kinase is required for growth of NIH/3T3 cells. Nature 1991;349:426–428.
6. Kyriakis JM, App H, Zhang X, Banerjee P, Brautigan DL, Rapp UR, Avruch J: Raf-1 activates MAP kinase-kinase. Nature 1992;358:417–421.
7. Howe LR, Leevers SJ, Gomez N, Nakielny S, Cohen P, Marshall CJ: Activation of the MAP kinase pathway by the protein kinase raf. Cell 1992;71:335–342.
8. Dent P, Haser W, Haystead TAJ, Vincent LA, Roberts TM, Sturgill TW: Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. Science 1992;257:1404–1407.
9. Ahn NG, Seger R, Bratlien RL, Diltz CD, Tonks NK, Krebs EG: Multiple components in an epidermal growth factor-stimulated protein kinase cascade. J Biol Chem 1991;266:4220–4227.
10. Gomez N, Cohen P: Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases. Nature 1991;353:170–173.
11. Crews CM, Alessandrini A, Erikson RL: The primary structure of MEK, a protein kinase that phosphorylates the *ERK* gene product. Science 1992;258:478–480.
12. Sturgill TW, Ray LB, Erikson E, Maller JL: Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. Nature 1992;334:715–718.
13. Lin L-L, Wartmann M, Lin AY, Knopf JL, Seth A, Davis RJ: cPLA2 is phosphorylated and activated by MAP kinase. Cell 1993;72:269–278.
14. Nemenoff RA, Winitz S, Qian N-X, Van Putten V, Johnson GL, Heasley LE: Phosphorylation and activation of a high molecular weight from of phospholipase A2 by p42 microtubule-associated protein 2 kinase and protein kinase C. J Biol Chem 1993;268:1960–1964.
15. Janknecht R, E.H. W, Pingoud V, Nordheim A: Activation of ternary complex factor Elk-1 by MAP kinase. EMBO J 1993;12:5097–5104.
16. Gille H, Sharrocks AD, Shaw PE: Phosphorylation of transcription factor $p62^{TCF}$ by MAP kinase stimulates ternary complex formation at c-*fos* promoter. Nature 1992;358:414–416.
17. Abdel-Hafiz HA, Heasley LE, Kyriakis JM, Avruch J, Kroll DJ, Johnson GL, Hoeffler JP: Activating transcription factor-2 DNA-binding activity is stimulated by phosphorylation catalyzed by p42 and p54 microtubule-associated protein kinases. Mol Endo 1992;6:2079–2089.
18. Pulverer B, Kyriakis JM, Avruch J, Nikolakaki E, Woodgett JR: Phophorylation of c-jun mediated by MAP kinases. Nature 1991;353:670–674.
19. Zhang X-f, Settleman J, Kyriakis JM, Takeuchi-Suzuki E, Elledge SJ, Marshall M, Bruder JT, Rapp UR, Avruch J: Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature 1993;364:308–313.
20. Van Aelst L, Barr M, Marcus S, Polverino A, Wigler M: Complex formation between RAS and RAF and other protein kinases. Proc Natl Acad Sci USA 1993;90:6213–6217.
21. Warne PH, Viciana PR, Downward J: Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. Nature 1993;364:352–355.
22. Vojtek AB, Hollenberg SM, Cooper JA: Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell 1993;74:205–214.
23. Moodie SA, Willumsen BM, Weber MJ, Wolfman A: Complexes of Ras-GTP with Raf-1 and mitogen-activated protein kinase kinase. Science 1993;260:1158–1161.
24. Leevers SJ, Paterson HF, Marshall CJ: Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature 1994;369:411–414.
25. Stokoe D, Macdonald SG, Cadwallader K, Symons M, Hancock JF: Activation of Raf as a result of recruitment to the plasma membrane. Science 1994;264:1463–1467.
26. Bruder JT, Heidecker G, Rapp UR: Serum-, TPA-, and Ras-induced expression from Ap-1/Ets-driven promoters requires Raf-1 kinase. Genes Dev 1992;6:545–556.
27. Sellmayer A, Uedelhoven WM, Weber PC, Bonventre JV:

Endogenous non-cyclooxygenase metabolites of arachidonic acid modulate growth and mRNA levels of immediate-early response genes in rat mesangial cells. J Biol Chem 1991;266:3800–3807.
28. Bonventre JV: Phospholipase A_2 and signal transduction. J Am Soc Nephrol 1992;3:128–150.
29. Kim DK, Bonventre JV: Purification of a 100 kDa phospholipase A_2 from spleen, lung and kidney. Antiserum raised to pig spleen phospholipase A_2 recognizes a similar form in bovine lung, kidney and platelets, and immunoprecipitates phospholipase A_2 activity. Biochem J 1993;294:261–270.
30. Williams NG, Roberts TM, Li P: Both p21ras and pp60v-src are required, but neither alone is sufficient, to activate the Raf-1 kinase. Proc Natl Acad Sci USA 1992;89:2922–2926.
31. Janknecht R, De Martynoff G, Lou J, Hipskind RA, Nordhem A, Stunnenberg HG: Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus. Proc Natl Acad Sci USA 1991;88:8972–8976.
32. Force T, Bonventre JV, Heidecker G, Rapp U, Avruch J, Kyriakis JM: Enzymatic characteristics of the c-Raf-1 protein kinase. Proc Nat Acad Sci USA 1994;91:1270–1274.
33. Kato J: Arachidonic acid as a possible modulator of estrogen, progestin, androgen, and glucocorticoid receptors in the central and peripheral tissues. J Steroid Biochem 1989;34:219–227.
34. Saltarelli MD, Yamada K, Coyle JT: Phospholipase A_2 and ^{3}H-hemicholinium-3 binding sites in rat brain: a potential second-messager role for fatty acids in the regulation of high-affinity choline uptake. J Neurosci 1990;10:62–72.
35. Ordway RW, Walsh JV, Jr, Singer JJ: Arachidonic acid and other fatty acids directly activate potassium channels in smooth muscle cells. Science 1989;244:1176–1179.
36. Ordway RW, Singer JJ, Walsh Jr. JV: Direct regulation of ion channels by fatty acids. Trends Neurosci 1991;14:96–100.
37. Kim D, Lewis DL, Graziadei L, Neer EJ, Bar-Sagi D, Clapham DE: G-protein $\beta\gamma$-subunits activate the cardiac muscarinic K^+ channel via phospholipase A_2. Nature 1989;337:557–560.
38. Kim D, Clapham DE: Potassium channels in cardiac cells activated by arachidonic acid and phospholipids. Science 1989;244:1174–1176.
39. Kim D, Duff RA: Regulation of K^+ channels in cardiac myocytes by free fatty acids. Circ Res 1990;67:1040–1046.
40. Hwang T-C, Guggino SE, Guggino WB: Direct modulation of secretory chloride channels by arachidonic and other cis unsaturated fatty acids. Proc Natl Acad Sci USA 1990;87:5706–5709.
41. Anderson MP, Welsh MJ: Fatty acids inhibit apical membrane chloride channels in airway epithelia. Proc Natl Acad Sci USA 1990;87:7334–7338.
42. Gerzer R, Brash AR, Hardman JG: Activation of soluble guanylate cyclase by arachidonic acid and 15-lipoxygenase products. Biochim. Biophys. Acta 1986;886:383–389.
43. Hansson A, Serhan CN, Haeggström J, Ingelman-Sundberg M, Samuelsson B: Activation of protein kinase C by lipoxin A and other eicosanoids. Intracellular action of oxygenation products of arachidonic acid. Bichem Biophys Res Commun 1986;134:1215–1222.
44. McPhail LC, Clayton CC, Snyderman R: A potential second messenger role for unsaturated fatty acids. Activation of Ca^{2+}-dependent protein kinase. Science 1984; 224:662–665.
45. Tsai M-H, Yu C-L, Stacey DW: A cytoplasmic protein inhibits the GTPase activity of H-Ras in a phospholipid-dependent manner. Science 1990;250:982–985.
46. Piomelli D, Wang JKT, Sihra TS, Nairn AC, Czernik AJ, Greengard P: Inhibition of Ca^{2+}/calmodulin-dependent protein kinase II by arachidonic acid and its metabolites. Proc Natl Acad Sci USA 1989;86:8550–8554.
47. Tsai M-H, Yu C-L, Wei F-S, Stacey DW: The effect of GTPase activating protein upon Ras is inhibited by mitogenically responsive lipids. Science 1989;243:522–526.
48. Zhao Z, Shen S-H, Fischer EH: Stimulation by phospholipids of a protein-tyrosine-phosphatase containing two src homology domains. Proc Natl Acad Sci USA 1993;90: 4251–4255.
49. Force T, Hyman G, Hajjar R, Sellmayer A, Bonventre JV: Noncyclooxygenase metabolites of arachidonic acid amplify the vasopressin-induced Ca^{2+} signal in glomerular mesangial cells by releasing Ca^{2+} from intracellular stores. J Biol Chem 1991;266:4295–4302.
50. Cai H, Erhardt P, Troppmair J, Diaz-Meco MT, Sithanandam G, Rapp UR, Moscat J, Cooper GM: Hydrolysis of phosphatidylcholine couples Ras to activation of Raf protein kinase during mitogenic signal transduction. Mol Cell Biol 1993;13:7645–7651.
51. Nakanishi H, Exton JH: Purification and characterization of the ζ isoform of protein kinase C from bovine kidney. J Biol Chem 1992;267:16347–16354.
52. Wartman M, Davis RJ: The native structure of the activated Raf protein kinase is a membrane-bound multisubunit complex. J Biol Chem 1994;269:6695–6701.