The 14.3.3 \( \zeta \) protein is a ubiquitous and abundant arachidonate-selective acyltransferase and putative phospholipase \( \Lambda \), which self-assembles into dimers and binds to c-Raf-1 and other polypeptides in vitro and in intact cells. The 14.3.3 polypeptides endogenous to SF9 cells associate in situ with both active and inactive recombinant Raf and copurify at a fairly reproducible molar ratio that is probably 1. Purified baculoviral recombinant Raf, despite its preassociated 14.3.3 molar ratio that is probably 1, binds additional recombinant 14.3.3 polypeptide in vitro, in a saturable and specific reaction, forming a complex that is resistant to 1 M LiCl. A two-hybrid analysis indicates that 14.3.3 \( \zeta \) binds primarily to Raf noncatalytic sequences distinct from those that bind Ras-GTP, and in vitro 14.3.3 \( \zeta \) binds to Raf without inhibiting the Ras-Raf association or Raf-catalyzed MEK phosphorylation. Deletion analysis of 14.3.3 \( \zeta \) (1-245) indicates that the 14.3.3 domain responsible for binding to Raf extends over the carboxyl-terminal 100 amino acids, whereas 14.3.3 dimerization is mediated by amino-terminal sequences. As with Ras, the 14.3.3 \( \zeta \) polypeptide does not activate purified Raf directly in vitro. Moreover, overexpression of recombinant 14.3.3 \( \zeta \) in COS cells beyond the substantial level of endogenous 14.3.3 protein does not alter endogenous Raf kinase, as judged by the activity of a cotransfected Erk-1 reporter. Coexpression of recombinant 14.3.3 with recombinant Myc-tagged Raf in COS cells does increase substantially the Raf kinase activity achieved during transient expression, which is attributable primarily to an increased level of Myc-Raf polypeptide, without alteration of Raf-specific activity or the activation that occurs in response to epidermal growth factor or 12-O-tetradecanoylphorbol-13-acetate. Nevertheless, evidence that 14.3.3 \( \zeta \) actively participates in Raf activation in situ is provided by the finding that although full-length 14.3.3 \( \zeta \) binds active Raf in situ, truncated versions of 14.3.3, some of which bind Raf polypeptide in situ nearly as well as full-length 14.3.3 \( \zeta \), are recovered in association only with inactive Raf polypeptides. Thus, 14.3.3 polypeptides bind tightly to one or more sites on c-Raf. Overexpression of 14.3.3 \( \zeta \) enhances the expression of recombinant Raf, perhaps by stabilizing the Raf polypeptide. In addition, Raf polypeptides bound to truncated 14.3.3 polypeptides are unable to undergo activation in situ, indicating that 14.3.3 participates in the process of Raf activation by mechanisms that remain to be elucidated.

An important insight into the initial step in Raf activation was the discovery that Raf binds directly to the GTP-bound form of Ras (1-3). This Raf-Ras-GTP interaction does not result directly in Raf activation, inasmuch as addition of Ras GTP to inactive, baculoviral recombinant Raf in vitro does not alter Raf kinase activity. Presumably, Ras GTP functions in situ to translocate Raf to the surface membrane so as to enable its activation by other processes. Support for this model is provided by the demonstration that fusion of plasma membrane targeting (CAAX) sequences onto the Raf carboxyl terminus is transforming and bypasses the need for Ras in Raf activation; a large increase in the activity of membrane-associated Raf is observed in growth factor-deprived cells, and EGF stimulates Raf CAAX activity a further 10-fold in a Ras-independent reaction (4, 5).

The inability of Ras to directly activate Raf, together with the finding that mitogen activation of Raf becomes Ras independent if Raf is targeted directly to the plasma membrane, implies that physiologic activation of (Ras bound) Raf requires Raf interaction with other plasma membrane components, e.g. lipids, polypeptides, or both. Ghosh et al. (6) reported that the Raf amino-terminal noncatalytic sequences bound to liposomes in a phosphatidylinerine-dependent reaction that is independent of Ca\(^{2+}\) and diacylglycerol. In this report, we describe the binding of c-Raf-1 in vitro and in situ to the 14.3.3 \( \zeta \) polypeptide, an arachidonate-selective acyl transferase and putative phospholipase \( \Lambda \) (7). We define the Raf domain employed for the binding of 14.3.3 \( \zeta \) in situ and the 14.3.3 \( \zeta \) domains necessary for self-association and Raf binding; we find that while carboxyl-terminal fragments of 14.3.3 bind Raf in situ nearly as well as full-length 14.3.3, only the latter is found in association with catalytically active Raf polypeptides in situ.

**MATERIALS AND METHODS**

cDNAs encoding murine 14.3.3 \( \zeta \) were isolated from a murine T cell DNA library by two-hybrid expression cloning according to Durfee et al. (8), using the c-Raf sequences 1-25/305-468 as bait (see “Results”). cDNAs encoding rat Erk-1, human MEK-1, and human C-Ha-Ras were expressed in Escherichia coli as GST\(^{1}\) fusion proteins using the p-GEX kg vector (9) and purified by glutathione-agarose affinity chromatography.

\(^{1}\) The abbreviations used are: GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; TPA, 12-O-tetradecanoylphorbol-13-acetate; EGF, epidermal growth factor.
phy. The free Erk-1 and 14.3.3ζ polypeptides were obtained after thrombin cleavage. Recombinant Raf polypeptide containing a hexahistidine tag at the carboxyl terminus was expressed in Sf9 cells using a recombinant baculovirus and purified by nickel chelate affinity chromatography. Active baculoviral Raf kinase was obtained by co-infection with baculoviruses encoding v-Ras and v-Src (10).

The transacylation activity of the recombinant 14.3.3ζ was measured according to Zupan et al. (7). The Raf kinase assay was performed as previously described (11, 12).

The binding in vitro of various polypeptides to GST or GST fusion proteins immobilized on glutathione-Sepharose was carried out at 30 °C for 30 min in buffer A containing 25 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol; polypeptide concentrations are described in the figure legends. The beads were washed in excess binding buffer three times; the retained polypeptides were eluted directly into SDS-containing buffer, separated by SDS-PAGE, and analyzed by protein staining, immunoblot, or autoradiography as described.

The association of polypeptides in situ was assessed during transient expression in COS M7 cells and transfected by the DEAE-dextran method. The cDNA sequences encoding Raf were inserted into two mammalian expression vectors: Myc-Raf contains a 33-amino acid epitope from human c-Myc, known to be reactive with the monoclonal antibody 9B7.3 (13), appended to the Raf amino terminus, and inserted into pMT2. Raf was also expressed as a GST fusion protein using the vector pEBG, which encodes glutathione S-transferase. The E. coli polypeptide/enhancer. The cDNA encoding 14.3.3ζ was introduced unmodified into the vector CMV5, into the pEB vector (lacking the glutathione S-transferase sequences) with a 9-amino acid epitope from the influenza hemagglutinin (HA epitope, Ref. 14) added to its carboxyl terminus, and into pEBG for expression in situ as a GST fusion. Deletion mutation of the 14.3.3ζ was made by polymerase chain reaction from the 5′- and 3′-ends of the cDNA. The polymerase chain reaction products were subcloned into the pEBG vector, and the structures were verified by DNA sequence analysis.

All transfections utilized a total of 20 μg of DNA; 48 h after transfection, extracts were prepared by homogenization in a buffer containing 25 mM Tris-Cl, pH 7.5, 1 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 25 mM p-glycerophosphate, 1 mM sodium vanadate, 1% Triton X-100, and proteinase inhibitors. Immunoprecipitations were carried out for 1 h at 4 °C using the monoclonal antibody 12CA5 for the HA epitope or the anti-Myc monoclonal antibody 9B7; immune complexes were harvested with protein G-Sepharose. GST-Raf and GST-14.3.3ζ fusions were recovered using glutathione-Sepharose beads. Immunoblots of Raf were carried out using 9B7.3 for Myc-Raf or a polyclonal antibody raised to cleaved purified recombinant murine 14.3.3ζ.

An antiserum against prokaryotic 14.3.3ζ was introduced as a GST fusion. Deletion mutation of the 14.3.3ζ was made by polymerase chain reaction from the 5′- and 3′-ends of the cDNA. The polymerase chain reaction products were subcloned into the pEBG vector, and the structures were verified by DNA sequence analysis.

All transfections utilized a total of 20 μg of DNA; 48 h after transfection, extracts were prepared by homogenization in a buffer containing 25 mM Tris-Cl, pH 7.5, 1 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 25 mM p-glycerophosphate, 1 mM sodium vanadate, 1% Triton X-100, and proteinase inhibitors. Immunoprecipitations were carried out for 1 h at 4 °C using the monoclonal antibody 12CA5 for the HA epitope or the anti-Myc monoclonal antibody 9B7; immune complexes were harvested with protein G-Sepharose. GST-Raf and GST-14.3.3ζ fusions were recovered using glutathione-Sepharose beads. Immunoblots of Raf were carried out using 9B7.3 for Myc-Raf or a polyclonal antibody raised to the carboxyl-terminal 12 amino acids of human c-Raf-1. Immunoblot of 14.3.3ζ was carried out using a polyclonal antibody raised to cleaved, purified recombinant murine 14.3.3ζ. The cDNA was expressed in Sf9 cells using a baculoviral vector encoding v-Ras and v-Src (10) identical to the rat ζ isoform of the 14.3.3ζ polypeptide, and that differed from a human platelet PLαζ polypeptide by a single conservative substitution (7). Inasmuch as 14.3.3ζ proteins have been reported to copurify or associate with a relatively large number of proteins (16), and 14.3.3ζ has been identified as a protein cofactor for a number of enzymes in vitro, including the ADP-ribosylation of Ras and other small GTPases by exoenzyme S (17), we examined several proteins other than Raf for their ability to associate with 14.3.3ζ in the two-hybrid system. No interaction of 14.3.3ζ with p70 S6 kinase and amino-terminal regulatory domain of protein kinase C ζ (residues 1–245) or c-Ha-Ras (1–185) was detected (data not shown). The relative selectivity of the 14.3.3-Raf association led us to undertake a further characterization of this interaction.

The two-hybrid method was employed to identify the region on Raf that interacts with 14.3.3ζ in comparison to MEK1 and Ras, proteins known to interact with Raf in a physiologic context. The Ras binding site has been previously localized to Raf residues 50–150 (3, 18), whereas neither MEK nor 14.3.3ζ interacts with Raf 1–257. MEK, a known Raf substrate, interacts strongly with the BXB-Raf and holo-Raf (1–648) but not at all with Raf 1–332 (Table I). By contrast, 14.3.3ζ interacts weakly with BXB-Raf and holo-Raf (1–648) but associates strongly with the Raf 1–332 (Table I). Thus, 14.3.3ζ binds in situ most avidly to a segment of Raf between amino acids 257 and 332, a noncatalytic region distinct from those that bind to Ras or MEK.

The binding of Raf to 14.3.3ζ in Vitro—The ability of recombinant Raf to interact directly with recombinant 14.3.3ζ was investigated. The 14.3.3ζ polypeptide was expressed in E. coli as a glutathione S-transferase fusion protein (9), purified by GSH affinity chromatography, and employed with and without thrombin cleavage (Fig. 1). The functional integrity of the recombinant 14.3.3ζ was evaluated by examining its ability to catalyze autoacylation from [14C]arachidonoyl sn-2-phosphatidylethanolamine. The GST-14.3.3ζ fusion protein and thrombin-cleaved 14.3.3ζ polypeptides, although devoid of PLαζ activity using a variety of substrates, each catalyze the cleavage of the sn-2 fatty acid of [14C]arachidonoyl sn-2-phosphatidylethanolamine and the formation of a covalent [14C]arachidonoyl-14.3.3ζ protein adduct, presumably an acyl-enzyme intermediate. Thus, the recombinant prokaryotic 14.3.3ζ exhibits an acyl transferase but not a PLαζ function, similar to that described by Gross and colleagues (7).

Raf 1 containing a hexahistidine tag at its carboxyl terminus was expressed in Sf9 cells by baculoviral infection and purified by nickel chelate chromatography, either as a catalytically inactive polypeptide (Fig. 2, lane 6) or in a catalytically active form, as a result of coinfection with baculoviral v-Ras plus v-Src (Fig. 2, lanes 1 and 3–5) (10). An antiseraum against prokaryotic recombinant murine 14.3.3ζ, reactive primarily with epitopes in the amino-terminal half of 14.3.3ζ (Fig. 5C, upper panel),
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**Fig. 2.** Copurification of 14.3.3 and c-Raf-1 from insect Sf9 cells. Sf9 cells were infected with baculovirus encoding human (His) 6-tagged c-Raf-1 alone or plus baculoviral encoded v-Ras and/or v-Src. The recombinant Raf was purified by nickel chelate affinity purification and subjected to SDS-PAGE prior to immunoblotting. Lane 1, Coomassie Blue stain of SDS-PAGE gel corresponding to the isolate shown in lane 5. Lanes 2–6, the upper part was immunoblotted with an anti-serum against carboxyl-terminal 12 amino acids of human c-Raf-1, and the lower part was immunoblotted with an antiserum against mouse 14.3.3 ζ. Lane 2, Sf9 cell extract without infection; lane 3, purified Raf confected with v-Src; lane 4, Raf confected with v-Ras; lane 5, Raf confected with v-Ras plus v-Src; lane 6, inactive baculoviral Raf; mwm, molecular weight markers.

readily immunoblots the 28- and 30-kDa 14.3.3 polypeptides endogenous to Sf9 (Fig. 2, lane 2) and COS cell (Fig. 7A, upper panel) extracts; immunoblot of several Raf-1 isolates (Fig. 2, lanes 2–6) shows that both the active (lanes 3–5) and inactive (lane 6) Raf polypeptides purified from Sf9 cells are recovered in association with endogenous Sf9 14.3.3 polypeptides. Both the recombinant Raf and Sf9 14.3.3 polypeptides are readily visualized on Coomassie Blue-stained gels of the preparations of purified Raf (Fig. 2, lane 1); although the relative Coomassie Blue binding per unit mass for c-Raf-1 and 14.3.3 is unknown, the comparable staining intensities observed for Raf and the co-purified 14.3.3, together with the general tendency of acidic polypeptides such as 14.3.3 to stain weakly with Coomassie Blue, suggests that the molar ratio of 14.3.3 to Raf in these isolates is at least 1. Moreover, a relatively constant ratio of two polypeptides is recovered in different isolates of Raf, whether active (Fig. 2, lanes 3–5) or inactive (Fig. 2, lane 6).

Despite the presence of considerable Sf9-derived 14.3.3 already bound to the baculoviral recombinant 14.3.3 polypeptide, such Raf preparations bind in vitro to a GST-14.3.3 ζ fusion protein but not to GST alone (Fig. 3, upper panel, lanes 1 and 2); preincubation of Raf with cleaved, purified E. coli recombinant 14.3.3 ζ polypeptide prevents the subsequent binding of Raf by immobilized GST-14.3.3 ζ, whereas preincubation of Raf with bovine serum albumin has no effect (Fig. 3, upper panel, lanes 1, 3, 4). The ability of 14.3.3 ζ to bind active baculoviral Raf was verified by the demonstration that GST-14.3.3 can specifically immobilize essentially all of the MEK phosphorylating activity in a preparation of active Raf (Fig. 3, lower panel, lanes 1–4), and the complex of active Raf and GST-14.3.3 is resistant to washing with 1 M LiCl. Extensive Raf autophosphorylation in vitro did not interfere with Raf binding to GST-14.3.3 ζ (Fig. 3, upper panel, lanes 6 and 7); inactive Raf polypeptide (identifiable by its slightly faster mobility in SDS-PAGE) also binds specifically to GST-14.3.3 ζ (Fig. 3, upper panel, lane 5).

These results demonstrate that baculoviral recombinant Raf, although purified as a complex with Sf9 14.3.3, binds additional 14.3.3 in vitro, and this binding is saturable. The ability of GST-14.3.3 to specifically adsorb essentially all the Raf polypeptides, both active (Fig. 3) and inactive, from such preparations indicates that GST-14.3.3 must bind Raf polypeptides that already contain bound Sf9 14.3.3. Alternatively, displacement or dimerization with the preassociated Sf9 14.3.3 is possible, the most plausible model envisions more than one binding site on Raf for 14.3.3. Thus, the ability of 14.3.3 ζ to interact with Raf in the two-hybrid yeast expression system certainly reflects the direct binding of the two polypeptides; however, the number of 14.3.3 binding sites on Raf and their precise localization remain to be more fully defined.

**Fig. 3.** Binding of Raf to GST-14.3.3 ζ in vitro. Upper panel, baculoviral Raf activated by coinfection with v-Ras and v-Src (~0.2 μM, lanes 1–4, 6, and 7) or unactivated (~0.3 μM, lane 5) and purified by nickel chelate chromatography was incubated with immobilized GST-14.3.3 ζ (20 μg/ml settled beads, lanes 1 and 3–6) or GST (20 μg/ml beads, lanes 2 and 7) directly (lanes 1, 2, and 5–7) or after preincubation with a 100-fold molar excess of purified 14.3.3 ζ (lane 3) or bovine serum albumin (lane 4). In lanes 6 and 7, the activated Raf was subjected to autophosphorylation in vitro in the presence of magnesium (10 mM), [γ-32P]ATP (100 μM, 30 °C for 30 min) prior to addition of GST-14.3.3 ζ (lane 6) or GST (lane 7). In lanes 1–5, Raf was detected by anti-Raf immunoblot; in lanes 6 and 7, 32P-Raf was detected by autoradiography. Lower panel, baculoviral Raf, activated in situ by coinfection with v-Ras and v-Src, was purified and mixed with GST-14.3.3 ζ (lanes 1 and 3) or GST (lanes 2 and 4) immobilized on GSH-Sepharose. Aliquots of the supernatants (lanes 1 and 2) and washed GSH-Sepharose beads (lanes 3 and 4) were assayed for Raf kinase activity by phosphorylation of GST-MEK (20 μg/ml) [γ-32P]ATP (0.1 mM, 2000 cpm/pmol).
evident as the 30-kDa Coomassie band seen just above Myc-tagged 14.3.3 polypeptides to the GST-14.3.3 fusions is although a free NH₂ terminus is not critical for 14.3.3 self-
lane 2

panel

bind to GSH-Sepharose (designated as expression of the GST-14.3.3 fusions reflects the variability in their ex-

FIG. 5

Coomassie Blue stain of the purified, COS recombinant GST-

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Table I

Interaction of Raf variants with 14.3.3 ζ, Ras and MEK in a yeast two-hybrid assay

| DNA binding domain fusion | Transactivation domain fusion |
|--------------------------|------------------------------|
| Full-length Raf (1–648)  | Light blue  | Blue  | Blue  |
| Raf (1–257)              | White       | White | Blue  |
| Raf (1–332)              | Blue        | White | Blue  |
| (1–25/305–648)          | Light blue  | Blue  | White |

Fig. 4. Association of recombinant Raf and 14.3.3 ζ in intact COS cells. Two cDNA encoding tagged Raf polypeptides were constructed, one in the vector pEB, encoding full-length Raf fused at its amino terminus to the carboxyl terminus of glutathione S-transferase, and a second in the vector pMT2 encoding a Myc epitope fused to the Raf amino terminus. In lanes 1–4, pEB encoding GST-Raf (lanes 1, 3, 4) or GST (lane 2) was cotransfected into COS cells with pMT2 encoding an HA-tagged 14.3.3 ζ (lanes 1–3) or the pMT2 HA vector (lane 4). Similarly in lanes 5–8, pMT2 encoding Myc-Raf (lanes 5, 7, 8) or pMT2 Myc vector (lane 6) was cotransfected with vector encoding HA-tagged 14.3.3 ζ (lanes 5–7) or empty pMT2 HA vector (lane 8). After 48 h, cells were extracted, and recombinant polypeptides were purified using GSH-Sepharose (lanes 1 and 2), anti-Myc monoclonal antibody 9B7.3 (lanes 5 and 6) for direct isolation of recombinant Raf, or anti-HA epitope monoclonal antibody 12CA5 for isolation of recombinant 14.3.3 ζ (lanes 3, 4, 7, and 8). Raf polypeptide in each isolate was detected by immunoblot with an anti-COOH-terminal Raf peptide antibody. Note that recovery of recombinant Raf using GSH-Sepharose (lane 1) or anti-HA immunoprecipitation (lane 5) is comparable to that achieved by immunoprecipitation of HA 14.3.3 ζ (lanes 3, 7).

polypeptide some endogenous 28-kDa 14.3.3 polypeptide, as visualized by immunoblot with anti-14.3.3 antiserum (not shown). Thus, a substantial portion of recombinant Raf is recovered in association with the coexpressed 14.3.3 ζ, indicating clearly that the 14.3.3 ζ associates in situ with most orallof the recombinant Raf-1 in COS cells.

The domains of 14.3.3 ζ, responsible for dimerization and Raf association were determined by coexpression in COS cells of Myc-Raf or Myc-14.3.3 with GST-14.3.3 (1–245) and a series of 14.3.3 fragments constructed as GST fusions (Fig. 5A). The transiently expressed GST-14.3.3 fusions were purified from COS cell extracts using GSH-Sepharose, and the isolates were evaluated for the presence of the cotransfected full-length Myc-tagged 14.3.3 polypeptide (Fig. 5B) or Myc-Raf (Fig. 5C). The Coomassie Blue stain of the purified, COS recombinant GST-14.3.3 fusions and their associated polypeptides is shown in Fig. 5B, upper panel. The variation in Coomassie Blue staining of the GST-14.3.3 fusions reflects the variability in their expression in situ rather than in their recovery, inasmuch as recovery of the irrelevant endogenous COS polypeptides that bind to GSH-Sepharose (designated as A and B, Fig. 5B, upper panel) is identical in all lanes. The binding of cotransfected Myc-tagged 14.3.3 polypeptides to the GST-14.3.3 fusions is evident as the 30-kDa Coomassie band seen just above band A in lane 2 (absent in lane 1) and more faintly in lanes 3–5. The identity of this band as Myc-14.3.3 is verified by anti-Myc immunoblot (Fig. 5B, lower panel). These results indicate that although a free NH₂ terminus is not critical for 14.3.3 self-association, the amino-terminal 80 amino acids of 14.3.3 are sufficient to confer some 14.3.3 self-association, and nearly full self-association is seen with GST-14.3.3 (1–140). Reciprocally, deletion of the amino-terminal amino acids from 14.3.3 markedly reduces its ability to self-associate; little (Fig. 5B, lower panel) is

Fig. 5. Deletion analysis of functional domains of 14.3.3 ζ. Truncations of 14.3.3 ζ were constructed by the polymerase chain reaction. The polymerase chain reaction products were subcloned to pEBG vector and expressed as GST fusions in COS cells. A, schematic diagram of GST-14.3.3 ζ variants. B, dimerization domain of 14.3.3 ζ, cDNAs encoding the GST-14.3.3 ζ variants shown in A were cotransfected with a vector encoding a full-length 14.3.3 ζ polypeptide tagged at its amino terminus with a Myc epitope. Extracts were prepared 48 h later; the GST fusion proteins were purified by GSH affinity chromatography and resolved on SDS-PAGE gel. A Coomassie Blue-stained gel is shown in the upper panel, and an immunoblot using anti-Myc antibody 9E10.2 is
Notably, only the GST-14.3.3 (1–245), polypeptide as GST-14.3.3 (1–245), the Raf association, inasmuch as the Myc-Raf bound to GST-14.3.3 (1–180) in situ probably cannot be attributed to a lack of 14.3.3 dimerization, inasmuch as the Myc-Raf bound to GST-14.3.3 (1–180) (Fig. 5C, middle panel, lane 5), which dimerizes quite well with Myc-14.3.3 (3) (Fig. 5B, bottom panel, lane 5), also lacks detectable kinase activity (Fig. 5C, bottom panel, lane 5). Taken together, these results establish that although the carboxyl-terminal 14.3.3 fragment 179–245, whereas no binding of Myc-Raf occurs to GST-14.3.3 (1–140) (Fig. 5C, middle panel, lane 4). Each of these GST-14.3.3 isolates was also assayed for the presence of active Raf kinase, estimated by its ability to phosphorylate and activate GST-MEK (Fig. 5C, bottom panel).

Effects of truncation on the ability of GST-14.3.3 to bind cotransfected Myc-Raf, the 14.3.3 amino-terminal 139 residues are largely dispensable; optimal Myc-Raf recovery is observed with GST-14.3.3 (139–245) (Fig. 5C, middle panel, lanes 2, 7), and considerable Myc-Raf is recovered with GST-14.3.3 (179–245) (Fig. 5C, middle panel, lane 8), whereas no binding of Myc-Raf occurs to GST-14.3.3 (1–140) (Fig. 5C, middle panel, lane 4). Notably, only the GST-14.3.3 (1–245), i.e. full-length 14.3.3, is associated with catalytically active Raf polypeptide. Thus, although GST-14.3.3 (139–245) retains as much Myc-Raf polypeptide as GST-14.3.3 (1–245), the Raf associated in situ with the truncated 14.3.3 is devoid of catalytic activity. The failure of active Raf to bind to truncated 14.3.3 polypeptides in situ probably cannot be attributed to a lack of 14.3.3 dimerization, inasmuch as the Myc-Raf bound to GST-14.3.3 (1–180) (Fig. 5C, middle panel, lane 5), which dimerizes quite well with Myc-14.3.3 (3) (Fig. 5B, bottom panel, lane 5), also lacks detectable kinase activity (Fig. 5C, bottom panel, lane 5). Taken together, these results establish that although the carboxyl-terminal 65 amino acids of 14.3.3 9 are sufficient to mediate association with Raf, only the full-length 14.3.3 sequences are found in association with catalytically active Raf polypeptides in situ.

Effect of 14.3.3 9 on Raf Kinase Activity—We sought to determine whether the binding of 14.3.3 9 to Raf directly alters Raf regulation or catalytic function. Bacterial recombinant Raf-1, activated in situ by coinfection with v-Ras plus v-Src, catalyzes a brisk autophosphorylation in vitro, as well as the phosphorylation of recombinant MEK1 in vitro. The recombinant 14.3.3 9 polypeptide at concentrations up to 3 9M is not phosphorylated at all by Raf, whereas GST MEK1 at 0.57 9M is phosphorylated by this amount of Raf kinase to an stoichiometry of 0.4 mol of PO4/mol of MEK (not shown) and activated to 25% of maximal. The 14.3.3 9 polypeptide does not inhibit Raf autophosphorylation when added at >100 molar excess to Raf, nor does 14.3.3 9 inhibit Raf-catalyzed MEK phosphorylation when present at 10–50-fold excess over MEK (not shown). Thus, 14.3.3 9 is neither a substrate nor an inhibitor of the Raf kinase in vitro.

We next examined the ability of 14.3.3 to modulate the activation of Raf kinase in vitro and in situ. An initial step in Raf activation in situ involves its binding to GTP-Ras. The effects of 14.3.3 9 polypeptide on the Ras-Raf interaction was assessed in vitro. The full-length Raf protein binds specifically to immobilized GST-Ras-GTP. Addition of 14.3.3 9 at >50-fold molar excess to Raf does not inhibit the binding of Raf to GST-Ras-GTP (Fig. 6, upper panel, lanes 1 and 2). In addition, after washing the immobilized GST-Ras-Raf complex, 14.3.3 9 is seen to have been retained by the GST-Ras, but only if Raf is present (Fig. 6, lower panel, compare lanes 1 and 2). These data demonstrate that 14.3.3 9 does not bind to Ras directly nor displace Raf from Ras, but rather it is capable of binding Raf in vitro so as to allow the formation of a ternary complex with Ras and Raf.

Addition of 14.3.3 9 to inactive Raf purified from SF9 cells does not activate Raf-catalyzed MEK phosphorylation (not shown). This negative result was obtained despite preincubation of Raf and 14.3.3 9 polypeptide in the presence of various combinations of GTP-Ras (both bacterial and baculoviral, fully processed Ras), phospholipid micelles prepared from bovine brain lipids, Mg2+ (10 mM), Ca2+ (0.1 mM), and ATP (100 mM), for 30 min prior to and after the addition of recombinant GST-MEK1. Inasmuch as a reliable in vitro assay for the activation of Raf kinase is not yet available and 14.3.3 and Raf associate in situ during transient expression, the influence of 14.3.3 9 on Raf activation was examined in situ by cotransfection. To examine the effects of 14.3.3 9 overexpression on the regulation of endogenous Raf (and other mitogen-responsive MEK activators), 14.3.3 was cotransfected in COS cells with an HA-tagged Erk-1 reporter. Overexpression of 14.3.3 9 several-fold above the already substantial level of endogenous 14.3.3 polypeptides did not alter recombinant Erk-1 activity in serum-deprived cells or in response to TPA or EGF (data not shown). We next examined the effects of 14.3.3 9 on the activity of recombinant Myc-Raf. COS cells transfected with Myc-Raf and various truncated forms of 14.3.3 9 were either serum deprived or stimulated with mitogens prior to harvest, and the Myc-tagged Raf-1 recombinant was immunoprecipitated and assayed for MEK kinase activity. Coexpression of Myc-Raf with GST-14.3.3 (1–245) increased the MEK kinase activity recovered in a Myc immunoprecipitate by about 2–3-fold, both in serum-deprived cells (Fig. 7A, bottom panel, compare lane 1 to
Identification of the 14.3.3 ζ Domains

Fig. 7. Effect of 14.3.3 ζ on the expression and activity of Myc-Raf in situ. Vectors encoding GST (lanes 1, 5, 9), GST-14.3.3 (1–245) (lanes 2, 6, 10), GST-14.3.3 (139–245) (lanes 3, 7, 11), or Raf (1–257) (lanes 4, 8, 12) were cotransfected with Myc-Raf (lanes 1–12) into COS cells; 48 h later, the cells were placed in medium containing 0.5% serum, and after a further 18-h incubation period, they were treated with carrier (lanes 1–4), EGF (10 ng/ml, lanes 5–8), or TPA (500 nm) (lanes 9–12) for 15 min, rinsed, and extracted. Aliquots of the cell extracts, normalized for protein content, were subjected to anti-Myc immunoprecipitaton (A, 3rd panel from top, and bottom panel) and GSH-Sepharose affinity chromatography (B, all three panels). A, the top panel shows an anti-14.3.3 immunoblot of the cell extracts. The panel lane 2) and in response to EGF (Fig. 7A, bottom panel, compare lane 5 to lane 6); EGF itself gave 2–3-fold activation of Myc-Raf (Fig. 7A, bottom panel, compare lanes 1 to lane 5). The ability of GST-14.3.3 to increase Myc-Raf activity is not due to Raf activation, as occurs with EGF or TPA, but appears to be attributable to an increased Myc-Raf polypeptide abundance, as seen by immunoblot of the whole cell extract (Fig. 7A, 2nd panel from top, compare lanes 1 to 2, 5 to 6, 9 to 10) and in the Myc immunoprecipitates (Fig. 7A, 3rd panel from top). Such an increase in Myc-Raf expression and recovery of activity was observed repeatedly on cotransfection with GST-14.3.3 (1–245) and was present but less pronounced with the truncated 14.3.3 GST fusion proteins that were capable of binding Myc-Raf in proportion to their somewhat lesser expression than GST-14.3.3 (1–245) (Fig. 7A, top panel and 2nd panel from top, compare lanes 2 to 3, 6 to 7, 10 to 11).

Based on the finding that GST-14.3.3 (139–245) bound Myc-Raf strongly but did not associate in situ with active Raf (Fig. 5C, lower panel), we examined whether GST-14.3.3 (139–245) could interfere with Myc-Raf activation. Fig. 7B demonstrates that although nearly equal amounts of Myc-Raf are recovered with GST-14.3.3 (1–245) and GST-14.3.3 (139–245) (Fig. 7B, middle panel), the latter is completely devoid of kinase activity (Fig. 7B, lower panel, lanes 3, 5, and 7). Nevertheless, whereas coexpression with GST-14.3.3 (1–245) increased Myc-Raf abundance (Fig. 7A, 2nd and 3rd panels from top) and activity (Fig. 7A, bottom panel) in parallel, the expression of GST-14.3.3 (139–245) had a lesser effect on Myc-Raf expression (Fig. 7A, 2nd and 3rd panels from top), and neither inhibited nor activated overall Myc-Raf activity (Fig. 7A, bottom panel). It is likely that the high levels of 14.3.3 endogenous to COS (and other cells) prevent the recombinant GST-14.3.3 (139–245) fragment from interfering with the activation of most Raf polypeptides, whereas those that become associated with GST-14.3.3 (139–245) are clearly excluded from the activation process. The inactive Raf polypeptides bound to GST-14.3.3 (139–245) were not activated by the addition of recombinant 14.3.3 ζ (1–245) in vitro (not shown).

DISCUSSION

The 14.3.3 class of 29–33-kDa polypeptides has been found to copurify with a broad array of proteins and has been repeatedly rediscovered as activators (e.g. of tyrosine hydroxylase), inhibitors (e.g. of protein kinase C), or cofactors (exoS) for a number of enzymes in vitro (16). Several recent reports (19, 20) have described an interaction of Raf with 14.3.3 polypeptides in vitro and in intact yeast similar to that described here. All studies of the Raf 14.3.3 interaction including the present report have employed one or both as recombinant polypeptides, and it is possible that the interaction observed in vitro, or even in situ examining the overexpressed recombinant polypeptides,
may not accurately reflect certain aspects of the interaction between the endogenous 14.3.3 and Raf polypeptides in situ. An apparent activation of recombinant human c-Raf-1 kinase in intact yeast occurs concomitant with overexpression of recombinant mammalian 14.3.3. Furthermore, disruption of the gene encoding the Saccharomyces cerevisiae 14.3.3 homolog, Bmh1, abrogates the ability of recombinant c-Raf-1 to rescue a Ste21-deficient strain containing a Raf-responsive mutant Ste7. Fantl et al. (21) found that microinjection or overexpression of 14.3.3 γ in Xenopus oocytes led to an increase in the activity of endogenous or recombinant Raf-1. Li et al. (22) reported that transient expression of 14.3.3 in NIH 3T3 cells had little effect on the activity of cotransfected reporters known to be responsive to active Raf-1; however, the ability of transiently expressed Raf-1 or BXB Raf-1 to activate these reporters was substantially augmented by cotransfection with 14.3.3. We find that although overexpression of 14.3.3 in cultured mammalian cells (COS or 293) has no effect on endogenous c-Raf-1 abundance or activity, measured directly or by the activity of a cotransfected Erk-1 reporter (not shown), coexpression of 14.3.3 with recombinant Myc-Raf results in greater Raf kinase activity (Fig. 7), much as found by Li et al. (22) Significantly, however, we find that this increase in Raf kinase activity is largely or entirely attributable to a 14.3.3-induced increase in the expression and abundance of recombinant Raf rather than an increase in Raf-1 specific activity. It seems probable that the ability of recombinant mammalian or endogenous yeast 14.3.3 to enhance the activity of mammalian Raf-1 when the latter is expressed in the heterologous milieu of S. cerevisiae or Xenopus oocytes may be attributable, in part or in whole, to an ability of 14.3.3 to enhance Raf-1 polypeptide abundance, perhaps e.g. by stabilizing the recombinant Raf-1 polypeptide.

Several observations nevertheless suggest that 14.3.3 may participate more directly in Raf-1 activation. Irie et al. (20) observed that addition of a maltose binding protein-14.3.3 fusion protein directly to inactive baculoviral Raf-1 or BXB Raf-1 to activate these reporters was substantially augmented by cotransfection with 14.3.3. In addition, 14.3.3 and Ras added directly to inactive baculoviral Raf do not alter Raf kinase activity, the further addition of a crude cellular extract results in a Ras-14.3.3-dependent, 2-3-fold activation of Raf-1 kinase. The biochemical mechanisms that underlie in vitro "activation" of Raf by 14.3.3 are not known, and the extent to which they reflect the ability of 14.3.3 to "stabilize" the Raf polypeptide in vitro, comparable to the effects that underlie the enhanced expression seen on cotransfection, is also not known. The present results, however, provide one persuasive piece of evidence that 14.3.3 participates in Raf activation beyond its ability to bind to and "stabilize" the Raf polypeptide. Carboxyl-terminal fragments of 14.3.3, which bind Raf nearly as well in situ as full-length 14.3.3 and which also provide some enhanced Raf expression in situ, are nevertheless recovered from cells in association only with catalytically inactive Raf polypeptides, whereas full-length 14.3.3 is recovered with catalytically active Raf kinase. This result suggests that the Raf-binding 14.3.3 fragments have lost a function critical to the activation of Raf. This function does not appear to be their ability to dimerize, inasmuch as the Raf polypeptides associated with GST-14.3.3 (1–180), which dimerizes normally (Fig. 5B, lane 5), are not catalytically active (Fig. SC, lane 5). We suggest that full-length 14.3.3 contributes to Raf activation either by recruiting an as yet unidentified polypeptide, by providing an intrinsic catalytic function, or both. The present data suggest that in addition to its ability to bind Raf concomitantly with Ras, the recombinant 14.3.3 polypeptide binds phospholipid and cleaves the sn-2 acyl bond. Whether the acyl transferase function of 14.3.3 is contributory to its role in the regulation of Raf function is not known. Thus, 14.3.3 polypeptides participate in the regulation of Raf activity; however, their specific biochemical function in Raf activation remains to be elucidated.

Subsequent to the completion of these studies, the structures of 14.3.3 γ (24) and γ (25) crystals were reported. Both 14.3.3 isoforms exhibited dimeric structures; each monomer was composed of nine helical segments arranged in antiparallel arrays. The dimer interface is created by highly conserved, primarily hydrophobic residues from the four amino-terminal helices, suggesting that 14.3.3 heterodimers will form readily. The carboxyl-terminal five helices of each monomer (which provide the Raf binding domain as demonstrated in the present report) are folded so as to provide within the dimer a cavity whose internal face is composed of (primarily hydrophobic) residues that are highly conserved in all 14.3.3 isoforms and whose external surface is provided by nonconserved residues. Future studies will determine the contributions of specific residues on each of these surfaces to the interactions of 14.3.3 with its polypeptide partners and phospholipids.

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