Associations of B- and C-Raf with Cholesterol, Phosphatidylserine, and Lipid Second Messengers

PREFERENTIAL BINDING OF Raf TO ARTIFICIAL LIPID RAFTS *

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The serine/threonine kinase C-Raf is a key mediator in cellular signaling. Translocation of Raf to membranes has been proposed to be facilitated by Ras proteins in their GTP-bound state. In this study we provide evidence that both purified B- and C-Raf kinases possess lipophilic properties and associate with phospholipid membranes. In the presence of phosphatidylinerine and lipid second messengers such as phosphatidic acid and ceramides these associations were very specific with affinity constants (K_D) in the range of 0.5–50 nM. Raf association with liposomes was accompanied by displacement of 14-3-3 proteins and inhibition of Raf kinase activities. Interactions of Raf with cholesterol are of particular interest, since cholesterol has been shown to be involved, together with sphingomyelin and glycerophospholipids in the formation of specialized lipid microdomains called rafts. We demonstrate here that purified Raf proteins have moderate binding affinity for cholesterol. However, under conditions of lipid raft formation, Raf association with cholesterol (or rafts) increased dramatically. Since ceramides also support formation of rafts and interact with Raf we propose that Raf may be present at the plasma membrane in two distinct microdomains: in raft regions via association with ceramide and ceramides and in non-raft regions due to interaction with phosphatidylinerine and phosphatidic acid. At either location Raf kinase activity was inhibited by lipid binding in the absence or presence of Ras. Ras-Raf interactions with full-length C-Raf were studied both in solution and in phospholipid environment. Ras association with Raf was GTP dependent as previously demonstrated for C-Raf-RBD fragments. In the presence of liposomes the recruitment of C-Raf by reconstituted Ras-farnesyl was only marginal, since almost 70% of added C-Raf was bound by the lipids alone. Thus Ras-Raf binding in response to activation of Ras-coupled receptors may utilize Raf protein that is already present at the membrane.

Stimulation of growth factor receptors activates Raf kinases via small GTP-binding proteins (Ras proteins) resulting in proliferation, differentiation, and cell survival (1, 2). The Raf kinase family consisting of A-Raf, B-Raf, and C-Raf shares three highly conserved regions: CR1, CR2, and CR3. The CR3 region represents the C-terminal catalytic domain, whereas CR1 contains Ras-binding domain (RBD) and a zinc-binding domain called cysteine reach domain (CRD). While C-Raf is a ubiquitously expressed protein with an apparent molecular weight (M_r) of 72,000–74,000, B-Raf (M_r 95,000) was found to be expressed preferentially in neuronal tissues and testis (3, 4). A-Raf, the smallest member with a M_r of 68,000, is limited in expression mainly to urogenital tissue. Cytosolic C-Raf exists as a 300–500 kDa large complex including heat shock and 14-3-3 proteins (5–7). Upon stimulation of cell surface receptors, C-Raf undergoes a series of activation events at the inner side of plasma membrane mediated by Ras and 14-3-3 proteins, including dephosphorylation and phosphorylation events (1, 2, 6).

Although Ras proteins play a crucial role in the activation of Raf the exact mechanism of Ras-Raf coupling is not completely understood. The RBD of C-Raf comprises residues 51–131 and binds directly to the so-called switch-I region of the activated Ras-GTP (8). A single point mutation in the Ras-RBD (R89L) abrogates Ras binding and blocks Raf activation (9). But not only RBD is involved in the Ras-Raf binding process. The CRD of C-Raf encompassing residues 139–184 also appears to be involved, although with lower binding affinity. Moreover, the interactions between CRD and fully processed Ras proteins have been proposed to be crucial for effective C-Raf activation (10, 11). Besides the ability to interact with processed Ras proteins, C-Raf-CRD was reported to possess binding sites for 14-3-3 proteins (12) in addition to the established C-Raf/14-3-3-binding sites at phosphoserine 259 and phosphoserine 621 (13, 14). The C-Raf-CRD was further proposed to bind to phospholipids at the plasma membrane. The first evidence that C-Raf-CRD interacts with phosphatidylinerine (PS) (15, 16) was

1 The abbreviations used are: RBD, Ras-binding domain; GPL, glycerophospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; SM, sphingomyelin; Gpp(NH)p, guanyl-5'-yl imidodiphosphate; mGppNHp, fluorescent analogue of Gpp(NH)p containing the fluorescent mant (m) group, N-methylanthraniloyl; GTPyS, guanosine 5'-O-(γ-thiotriophosphate); SA, streptavidin; CRD, cysteine reach domain; LUV, large unilamellar vesicles; far, farnesyl residue; OS, glutathione-S-transferase; ERK, extracellular signal-regulated kinase; MEK, mitogen and extracellular signal-regulated kinase; BSA, bovine serum albumin; PKC, protein kinase C; GDPyS, guanosine 5'-O-(α-thiodiphosphate); Ni-NTA, nickel-nitrilotriacetic acid.
not unexpected, since C-Raf-CRD exhibits a structure very similar to the C1-domain of protein kinase C family proteins (17, 18), although C-Raf-CRD does not bind tumor promoting phorbol esters and binds only weakly diacylglycerol (5). Taken together, C-Raf-CRD appears to have a complex and multifunctional role in the regulation of C-Raf kinase activity. Further investigations revealed (19) that the C-Raf kinase interacts not only with PS but also with phosphatidic acid (PA). In contrast to PS, the interaction sites for PA have been localized within the CR3 region of C-Raf kinase. Whereas the agonist-induced hydrolysis of phosphatidylcholine (PC) results in PA production, the cleavage of sphingomyelin (SM) leads to generation of ceramides. Both PA and ceramide are considered to act as intracellular lipid second messengers. Interactions between ceramides and C-Raf kinases have been observed (20, 21).

In the current study a quantitative approach for Raf associations with lipids has been performed using Biomolecular Interaction Analysis, a technology based on surface plasmon resonance, which allows monitoring of biomolecular interactions in real time. The newly developed L1-sensor chip permits capturing of intact lipid vesicles by hydrophobic residues localized on the chip surface (22). The vesicles captured in this way provide a chemically and physically stable environment which resembles cellular membranes.

The interactions of a number of signaling molecules with the plasma membrane depend not only on their association with particular lipids: a special architecture of some lipid microdomains called rafts plays an important role in the transduction of signals. Such lipid rafts form liquid-ordered phases in the lipid bilayer and are dispersed in the bulk of a liquid–disordered phase of the plasma membrane (23–26). These microdomains have been found to be highly enriched in cholesterol and sphingomyelin, making them resistant to solubilization with non-ionic detergents (for example, Triton X-100) thus yielding detergent-resistant membrane fractions (26). The special features of rafts are probably due to the tight packing of highly saturated fatty acid residues in sphingolipids with cholesterol. In contrast to rafts, the non-raft regions are composed mainly of glycerophospholipids (GPLs). Depending on cell type, detergent-resistant membranes may contain caveolin, a 21–24-kDa structural protein, which appears to mediate the formation of 60–100 nm flask-shaped invaginations (caveolae), which are distinct from the much larger (250–300 nm) clathrin-coated pits (25, 27) and may thus represent a subclass of rafts. Several other structural/integral proteins have been found to be associated with rafts, such as flotillins/reggies (28–30), stomatin (31), and the MAL/BENE proteolipids (32). Additionally, rafts (and caveolae) are highly enriched in components that mediate signal transduction and therefore are thought to increase the efficiency and specificity of signal transduction processes. Besides glycosylphosphatidylinositol-anchored proteins that associate with rafts at the extracellular side of the plasma membrane, several acylated (and non-acylated) proteins such as heterotrimeric and small G-proteins, Src kinases, eNOS, Shc, Nck, and MAPK have been found to be attached to the rafts/caveolae microdomains at the cytosolic side (25, 33–37). Moreover, several transmembrane receptors (tyrosine kinase receptors, heptahelical receptors, and T-cell antigen receptors) have been reported to be enriched in lipid rafts (26, 33). Less is known about the precise association of Raf kinases with lipid rafts. Although interactions of C-Raf with raft/caveolar fractions have been observed (35, 38) it is still unclear whether these interactions take place with lipid components or with caveolin, which is normally present in such preparations. Because of the small size (50 nm in average) and their flat structure, the visualization of rafts, in contrast to caveolae, is very difficult and was successfully performed only by fluorescence labeling, cross-linking (clustering) of raft proteins by antibodies and photonic force microscopy measurements (26, 39). Taken together, raft microdomains have been characterized as membrane regions consisting of a unique lipid environment and are believed to function in cellular signaling by forming platforms for individual receptor signaling complexes.

In this study we examined the associations of purified and functional full-length B- and C-Raf kinases with membrane lipids, particularly with phosphatidylcholine (PC), phosphatidylethanolamine (PE), PS, phosphatidylinositol (PI), PA, SM, ceramides, and cholesterol using reconstitution techniques and biosensor measurements. We report here that purified C-Raf binds tightly and specifically to PA, PS, ceramides, and cholesterol. In contrast, the association with PC, PE, PI, and SM was much less pronounced. Purified B-Raf exhibited binding properties similar to C-Raf. The interactions of C-Raf with cholesterol were examined under conditions which allow the formation of raft microdomains. In this case we monitored a significant increase of Raf association with cholesterol, indicating that raft formation potentiates Raf binding to cholesterol. In general, binding of active Raf kinases to liposomes led to a considerable decrease of kinase activity and displacement of 14-3-3 proteins. Ras-Raf interaction studies revealed that association of Raf with phospholipid vesicles containing farnesylated Ras was only marginally increased compared with vesicles without Ras. Based on these results we discuss an alternative model for Raf activation in which Raf recruitment by Ras involves primarily diffusion in the plane of the membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—The phospholipids (PC, PE, PI, and PA), SM, cholesterol, streptavidin, imidazole, benzamidine, leupeptin, aprotinin, CHAPS, and n-octyl-a-D-glucoside were obtained from Sigma. Nonidet P-40, GTP, GTPyS, GppNHp, GDP, and GDPβS were from Roche Molecular Biochemicals. Ceramides (natural sources) were purchased from Ma- treya (BIO-TRENDS) and biotin-PE was from Molecular Probes. Sepha- rose CL-4B and glutathione-Sepharose were obtained from Amersham Biosciences. Ras and Raf antibodies were obtained from Transduction Laboratories, polyclonal anti-13-3-3 antibodies were from Santa Cruz Biotecnology and monoclonal anti-phospho-ERK1/2 antibodies were from New England Biolabs. Horseradish peroxidase-conjugated polyclonal anti-rabbit and anti-mouse IgG were purchased from Amersham Biosciences.

**Cell Culture, Protein Purifications, SDS-PAGE, and Western Blot Analysis**—For the production of recombinant Raf kinases, SF9 cells were infected with baculoviruses at the multiplicity of infection of 5 and incubated for 48 h at 30 °C. The cells were then washed with phosphate-buffered saline and pelleted at 230 × g. The SF9 cell pellets (2 × 10^8) were lysed in 10 ml of Nonidet P-40 lysis buffer containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM Na-pyrophosphate, 25 mM β-glycerophosphate, 25 mM NaF, 10% glycerol, 0.75% Nonidet P-40, and a mixture of standard protease inhibitors (1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) for 45 min with gentle rotation at 4 °C. The lysate was centrifuged at 27,000 × g for 30 min at 4 °C. The supernatants (10 ml) containing GST-tagged Raf kinases were incubated with 0.5 ml GS beads for 2 h at 4 °C with rotation. After incubation the GS beads were washed 3 times with Nonidet P-40 buffer, whereby the third wash contained only 0.2% Nonidet P-40 instead of 0.75%. The Raf kinases bound to the beads were eluted 3 times with 0.5 ml of 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 25 mM β-glycerophosphate, 25 mM NaF, 10% glycerol, 0.1% Nonidet P-40, and 20 mM glutathione. The purification procedure for His-tagged Raf kinases was similar as described above with the exception that the SF9 cell lysates (10 ml) were incubated with 0.5 ml of Ni-NTA-agarose. The bound proteins were then eluted with imidazole using a step gradient. The purity of the Raf kinase preparations was documented by SDS-polyacrylamide gel electrophoresis (10% gels) and staining with Coomassie Blue (see Fig. 1A). For Western blot analysis the gels were transferred to nitrocellulose membranes (Schleicher & Schuell) and probed with antibodies specific for C-Raf (78), B-Raf (77), Ras, 14-3-3 proteins and phospho-ERK. After washing,
the membranes were incubated with specific secondary horseradish peroxidase-conjugated antibodies and detected by enhanced chemiluminescence (ECL, Amersham Biosciences).

Expression and purification of H-Ras were performed as described before (40). Isoprenylation of full-length H-Ras protein was carried out with purified farnesyltransferase and purified geranylgeranyltransferase I, using 0.3 nmol of purified proteins. 0.5 nmol of H-Ras was incubated with 20 nmol of farnesyltransferase and 1 nmol of farnesyl pyrophosphate in 30 mM Tris-HCl, pH 7.4, 50 mM NaCl, 20 μM ZnCl₂, 5 mM octylglycoside, and 2 mM diithioerythritol in a total volume of 5 ml for 3 h at 30 °C. Farnesylated product was separated from non-farnesylated H-Ras by extraction with Triton X-114 (42, 43). The excess of detergent was removed by precipitation of the detergent with 2× volume of 50% ethanol. The precipitate was collected in the void volume (300 ml) and was further concentrated by Speed vac apparatus. To visualize the associated Raf kinase with lipids covalently labeled with mant-GppNHp, the concentrated samples were applied to SDS-PAGE (10% gels), transferred to nitrocellulose membranes, and incubated with specific antibodies. After detection of lipid bound Raf by enhanced chemiluminescence (ECL) the quantification was performed by scanning laser densitometry.

Kinase Activity Measurements—Kinase assays with Raf proteins were performed using recombinant MEK and ERK-2 as substrates in 25 mM Hepes, pH 7.6, 150 mM NaCl, 25 mM β-glycerolphosphate, 10 mM MgCl₂, 1 mM dithiothreitol, and 1 mM Na vanadate buffer (50 μl final volume) with 0.5 μg of purified Ras proteins at 4 °C overnight. The kinase assays, the sensor chip surface was regenerated by injection of 20 mM CHAPS followed by washing with biosensor buffer before reinjecting a new sample. The subtraction of sensorgrams was calculated from non-farnesylated H-Ras by extraction with Triton X-114 (42), followed by removal of the detergent with 2× volume of 50% ethanol. The precipitate was collected in the void volume (300 ml) and was further concentrated by Speed vac apparatus. To visualize the associated Raf kinase with lipids covalently labeled with mant-GppNHp, the concentrated samples were applied to SDS-PAGE (10% gels), transferred to nitrocellulose membranes, and incubated with specific antibodies. After detection of lipid bound Raf by enhanced chemiluminescence (ECL) the quantification was performed by scanning laser densitometry.

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Purification and Characterization of Raf kinases—Analogous to the binding of protein kinase C to lipids, such as PS and diacylglycerol, C-Raf was shown to possess binding affinity for PS but revealed almost no binding to diacylglycerol (5, 15, 16). Additionally C-Raf was found to bind to PA (19). Interactions of B-Raf with membrane lipids have not been reported so far. Since most published results, including Ras-Raf binding assays (43, 49), were performed either with fragments of C-Raf or with C-Raf expressed and purified from bacterial origin, a material which turned out to be non-functional with regard to kinase activity (Fig. 1B),2 we expressed and purified diverse Raf kinases from SF9 insect cells. Contrary to the bacterial expression system, Raf kinases expressed in SF9 cells possess important co-translational modifications such as phosphorylation of serine 621 and serine 259 of C-Raf (1, 50), thus allowing associations of rat constant. The affinity constant K<sub>d</sub> was calculated from the equation K<sub>d</sub> = k<sub>a</sub>/k<sub>d</sub>.

RESULTS

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2 U. R. Rapp, unpublished data.
The constitutively active variant of C-Raf (Y340D/Y341D) had approximately 12-fold higher activity than C-Raf wt, whereas the kinase dead mutant of C-Raf (K375W) and C-Raf wt from E. coli did not show any kinase activity. The BXB form of C-Raf displayed elevated activity relative to wild type C-Raf that was further increased (approximately 20-fold) in the BXB-Y340D/Y341D mutant. In comparison to C-Raf, the B-Raf exhibited ~110-fold higher kinase activity.

**Fig. 2.** Ras binds to full-length C-Raf in GTP-dependent manner. GST-C-Raf wt immobilized to GS (60 nm) was incubated with purified Ras proteins at 40 nm (lanes 1 and 3) and 200 nm (lanes 2 and 4) final concentrations, respectively. Immobilized GST-C-Raf-BXB was incubated with 200 nm H-Ras. Ras proteins were loaded either with GTPγS (depicted as T) or GDP (depicted as D). Following SDS-PAGE and immunoblotting, Ras and Raf were visualized by specific antibodies and ECL.
the possible C-Raf recruitment by Ras in vitro, we reconstituted farnesylated H-Ras into the phospholipid vesicles and measured the Raf recruitment by Ras. As reported (51), farnesylated Ras reveals much higher association rates to phospholipid monolayers than non-farnesylated Ras. We have obtained similar results using our LUV preparations. As shown in Fig. 4A the association of farnesylated Ras to liposomes was 4-fold higher compared with non-farnesylated Ras. In these experiments the molar ratio of Ras to phospholipids was 1:2.400 corresponding to a weight ratio of 1:240. Next we investigated the association of purified full-length C-Raf and farnesylated Ras in the presence of liposomes, thus mimicking the normal constellation of these interactions (Fig. 4, B and C). For these experiments we have chosen a high excess of farnesylated Ras over C-Raf and omitted PS and PA in the lipid composition of vesicles. But even under such conditions the recruitment of Raf by Ras was marginal, indicating that the recruitment of Raf by lipids plays a primary role. In the experiments using LUVs which contained additionally PS and PA, i.e. vesicles which correspond to the composition of the inner leaflet of the plasma membranes, it was difficult to register any additional recruitment of Raf by Ras (data not shown).

Association of Raf Kinases with Phospholipids and Lipid Second Messengers—Since the extent of Raf recruitment by Ras under in vitro conditions described in Fig. 4 was comparable with that caused by simple Raf-lipid associations, we hypothesized that Raf translocation to the lipid surfaces might be independent of activated Ras. To examine this we characterized the interactions of purified Raf kinases with phospholipids such as PC, PE, PS, PI, SM, and cholesterol and lipid second messengers PA and ceramide. To assess whether these associations take place with purified full-length and functional B- and C-Raf kinases, we developed a lipid binding assay using phospholipid vesicles prepared by the extrusion method (47) and tested Raf kinases such as B-Raf, C-Raf, and constitutively active forms of C-Raf. The results from Raf-lipid association experiments are depicted in Fig. 5. Since we originally observed that Raf kinases do not exhibit significant association to vesicles composed of PC, PE, and SM we used vesicles with a lipid ratio of PC/SM/PE = 50:13:37 (mol %) as a basic lipid mixture. Vesicles with other lipid combinations containing additionally PS, PA, PI, or ceramides (20 and 30% for PS, 10 and 20% for PA, PI, and ceramides, respectively) have been prepared from basic PC/PE/SM vesicles by substitution of PC content with lipids of interest. The interactions of C-Raf with cholesterol are shown in Fig. 9. The basic lipid mixture enriched by 20–30% PS represents the typical lipid composition of the inner leaflet of plasma membranes (52). Additionally, the PS content may vary depending on the structure of membrane microdomains such as rafts or caveolae (25, 26, 53). The results shown in Fig. 5, A and B, provide direct evidence that C-Raf preferentially associates with PS and PA and to somewhat lower extent with ceramide. The constitutively active mutant C-Raf-Y340D/Y341D exhibited the same binding pattern as C-Raf wt (Fig. 5C). Control proteins such as purified GST alone and ERK-1 (see Fig. 5B)
displayed only marginal binding to the vesicle samples tested without any specificity for particular lipids. The same was true when Raf proteins were added in the absence of lipid vesicles. The C-Raf-BXB, a protein which represents the C-terminal half of C-Raf, was still capable of association with PS and PA, but failed to interact with ceramides (Fig. 5D). Of particular interest are the experiments with regard to associations of B-Raf with membrane lipids, since such interactions have not been investigated previously. We found that B-Raf-like C-Raf possesses high binding affinity for PS and PA (Fig. 5E), yielding 60–70% lipid-associated Raf. In contrast, unlike C-Raf, B-Raf showed increased association with vesicles containing PI, indicating that B-Raf does not discriminate between the acidic phospholipids tested. In parallel to the specific binding effects of Raf proteins to lipids, we observed that interactions of active Raf kinases with liposomes resulted in a considerable reduction of initial kinase activity. As documented in Fig. 5, C and D, the reconstitution of constitutively active C-Raf-Y340D/Y341D and C-Raf-BXB into liposomes resulted in more than 90% reduction of initial kinase activity. However, the decrease of B-Raf kinase activity in the presence of liposomes was not as dramatic as that detected for C-Raf (Fig. 5E). Only in the presence of PS containing vesicles was a strong activity decrease (down to 3% of initial) observed, in the presence of other lipid combinations the residual activity remained at about 30% of initial values. Taken together, the experiments shown in Fig. 5 illustrate clearly that the functionally intact and full-length B- and C-Raf kinases associate with membrane lipids, preferentially with PS and PA and to somewhat lower degree with ceramide.

Raf-Lipid Interactions Monitored by Biosensor Technology—To derive kinetics and equilibrium parameters for the interactions between Raf kinases and lipid vesicles, we applied Biomolecular Interaction Analysis technology using the newly developed L1 sensor chip, which permits capturing of lipid vesicles by hydrophobic anchors. The injection of phospholipid vesicles at 0.4 mM resulted in approximately 4,000 resonance units. In general, there were no significant differences in the binding properties of vesicles tested to the sensor chip surface. To characterize the Raf interactions with lipids in terms of quantitative kinetic studies, we re-examined the results presented in Fig. 5 using biosensor technology. To assess kinetic parameters and equilibrium constants for Raf-lipid interactions, it was necessary to monitor the specific ingredients of this protein-lipid association process. For that purpose the control flow cell was loaded with the basal lipid mixture (PC/PE/SM vesicles) while the second flow cell was loaded with vesicles containing additionally PS, PA, PI, ceramide, or cholesterol (for cholesterol binding results, see Fig. 9). As shown in Fig. 6 the binding pattern of C-Raf with lipids was very similar to those obtained by the simple lipid-binding assay depicted in Fig. 5. However, the extent of C-Raf binding to PS and PA using biosensor methodology was considerably greater compared with results obtained by standard lipid binding assays. A sitespecific peptide derived from the sequence of C-Raf (amino acids 389–408) representing the putative binding domain for PA was synthesized and revealed high affinity for PA containing vesicles in biosensor measurements, in contrast to numerous control peptides tested (data not shown). These data confirm the assumption that PA binding by C-Raf indeed occurs within the kinase domain (CR3).

Kinetic Analysis—To evaluate the kinetic and equilibrium parameters for associations between C-Raf kinases and lipids, the measurements have been performed in the presence of increasing concentrations of C-Raf. The apparent association ($k_\text{a}$) and dissociation rate constants ($k_\text{d}$) were evaluated from the differential binding curves (Fc2–Fc1). Representation association-dissociation curves for interactions of C-Raf to lipids are illustrated in Fig. 7. The corresponding data for $k_\text{a}$, $k_\text{d}$, and $K_D$ values are summarized in the Table I. The association of C-Raf to PA appear to be most tight with an apparent $K_D$ value of approximately 0.5 nM followed by PS with $K_D$ = 12 nM and ceramide with $K_D$ = 52 nM. These results document the high affinity and specificity of C-Raf-lipid interactions under the conditions used in our binding studies. The very slow dissociation of bound Raf kinase to lipids may be explained in part by re-association events and in this case the calculated $k_d$ values should be considered as a first approximation. Such effects can be reduced by increasing the flow rate or lowering the lipid density. However, neither elevation of flow rate to 200 µl/min nor injection of highly concentrated NaCl buffer (3 M) or reduction of lipid concentrations from 0.4 mM to 80 µM changed significantly the dissociation properties. Injections of 10 mM NaOH yielded only incomplete removal of bound Raf. Therefore the bound lipid vesicles were cleared by injection of CHAPS or octylglucoside after each measurement.

Association of Raf Kinases with Liposomes Leads to Displacement of 14-3-3 Proteins—In parallel to the specific Raf-lipid interactions and loss of kinase activity as demonstrated in Fig. 5, we observed in the presence of liposomes an almost complete dissociation of 14-3-3 proteins. The displacement of 14-3-3 proteins upon Raf association with LUVs was observed with both C-Raf and B-Raf. A typical experiment is shown in Fig. 8. The preparations of both C-Raf wt and B-Raf wt contained 14-3-3 proteins (lanes 6 and 7 in Fig. 8). After addition and incubation with PS/PA-containing vesicles, the liposome-bound Raf was separated from the free Raf. As evident from Fig. 8 the Raf fraction associated with liposomes has almost quantitatively lost the 14-3-3 proteins (lane 1 in Fig. 8). Preincubation of phospholipid vesicles with H-Ras proteins (both farnesylated and non-farnesylated forms were used) did not prevent 14-3-3 displacement (lanes 2–5 in Fig. 8). Based on these data we propose that the displacement of 14-3-3 proteins may be a consequence of lipid interactions with the putative third 14-3-3-binding site within the CRD region of Raf. These observations on 14-3-3 displacement together with the observed loss of kinase activity of lipoph-bound Raf are in agreement with results of Tzionov et al. (54), who reported that displacement of 14-3-3 proteins from activated C-Raf by addition of a synthetic phosphopeptide corresponding to the pS-621-binding region was also accompanied by significant reduction of Raf kinase activity.

Raf Associates with Cholesterol and Lipid Rafts—to mimic the association of signaling molecules with rafts, we prepared liposomes containing variable amounts of cholesterol, SM, and GPLs. To test the general binding properties of Raf for cholesterol, we first took advantage of the Raf-lipid binding assay presented in Fig. 5 using the Sepharose CL-4B column method. We created two sets of lipid vesicles (in the presence of either 13 or 26% SM) and with increasing amounts of cholesterol (2, 10, and 20%, and 40 mol %). The concentration of PE was kept constant while PC was substituted for cholesterol as indicated. As depicted in Fig. 9A, C-Raf wt exhibited moderate and concentration-dependent binding for cholesterol. In the presence of 13% SM (left panel), 35–40% of added C-Raf was retained by cholesterol (a 10% PA control showed retention of about 60% C-Raf). To achieve lipid compositions favoring raft formation (55, 56), SM content was increased to 26% (right panel). In the presence of 10 and 20% cholesterol, the extent of Raf association with liposomes was comparable with those observed in the left panel. Strikingly, in the presence of 40% cholesterol Raf association with liposomes increased up to 75–80% of added Raf (Fig. 9A). In preliminary experiments B-Raf exhibited sim-
lipid vesicles (1.2 mM) consisting of lipid compositions indicated in Table 1. We measured the maximal responses measured for specific C-Raf interactions with liposomes. Data represent mean ± standard deviations from two independent experiments.

Similar binding properties for cholesterol (data not shown). Next we evaluated the same interactions depicted in Fig. 9A using the biosensor technique, since the background binding of analyte (Raf) to the captured control vesicles amounts to less than 0.1% of injected Raf using this technique. The results performed by the surface plasmon resonance technique correlated very well with results obtained by column binding assays with the exception that Raf binding in the presence of 10% cholesterol was almost not detectable. This could be explained by the fact that the surface plasmon resonance technique records the initial association of Raf in contrast to the equilibrium conditions used in the Sepharose CL-4B experiments. Under the conditions of raft formation (Fig. 9, C and D), liposomes containing 26% SM and 40% cholesterol the association of Raf increased dramatically from 2-fold to almost 9-fold over the background binding. These results indicate (i) that a special composition and proportion of lipids (cholesterol/SM/GPL) is required for raft formation and (ii) that full-length wild type Raf exhibits high association tendency for cholesterol in raft-like microdomains. Additionally, we examined the effects of simultaneous incorporation of cholesterol and ceramides into the vesicles, since metobolical formation of ceramide from SM is supposed to occur within the lipid rafts (57). Interestingly, already small amounts of ceramide (5 mol %) considerably stabilized the raft structure (Fig. 9). These results indicate that ceramide may substitute for cholesterol and that C-Raf binds to both cholesterol and ceramides in rafts. The association-dissociation behavior of C-Raf binding to liposomes containing cholesterol is shown in Fig. 9, B and C. Similar to the results obtained with PS- and PA-containing vesicles (Fig. 7), C-Raf exhibits a very slow dissociation, indicating a tight association of Raf to cholesterol. The functional consequences of Raf interactions with cholesterol (and rafts) were examined by measuring the kinase activity of C-Raf wt (basal activity) and constitutively active mutant C-Raf-Y340D/Y341D in the presence of liposomes listed in Fig. 9. We observed, similar to the results shown in Fig. 5, that Raf kinase activity was considerably decreased in the presence of cholesterol containing vesicles (data not shown). These data are in accordance with the observation that diverse signaling molecules such as Ras, Src kinases, and eNOS are maintained in their inactive form during the association with rafts/caveolae (25, 35).

**DISCUSSION**

In this report we provide evidence that purified B- and C-Raf kinases possess a high tendency for associations with phospho-
and quantified by scanning laser densitometry. The amounts of Raf and 14-3-3 were loaded directly with 5 and 10 pmol of C-Raf and B-Raf, respectively. Similar results have been obtained from five independent experiments.

The phospholipid vesicles (1.2 nm) consisting of PC, SM, PE, PS, and PA (20:13:37:20:10 mol %) were incubated with farnesylated and non-farnesylated H-Ras (120 pmol), which were loaded either with GTP·S or GDP (lanes 2–5) or without addition of Ras (lane 1). Purified C-Raf (10 pmol, A) or B-Raf (10 pmol, B) were added and the mixture was further incubated for 30 min at 4 °C. The lipid-bound Raf was separated from the free Raf by Sepharose CL-4B columns (2.5 ml). The opalescent vesicle fractions were applied to SDS-PAGE and immunoblotted. The amounts of Raf and 14-3-3 were confirmed with full-length C-Raf expressed in mammalian cells and that Raf possesses a high tendency to associate with lipid environment. Therefore we investigated in more detail, using the reconstitution technique, the putative interactions of Raf kinases with lipids.

Specific Associations of B- and C-Raf Kinase with PS, PA, and Ceramides—Evidence that fragments of Raf proteins, particularly C-Raf-CRD, interact with phospholipids have been presented (15, 16). The amino acids in C-Raf-CRD interacting with PS have been mapped as Arg-143, Lys-144, and Lys-148, since substitution of all three basic residues to alanine resulted in a C-Raf variant that failed to interact with PS. Additionally, further interactions between Raf and membranes might occur by insertions of hydrophobic residues of C-Raf-CRD into the lipid bilayer. A similar mechanism for membrane attachment has been proposed for Rabphilin-3A, which is an effector of the Ras-related small GTPase Rab3A (17, 60). Based on x-ray crystal structure, the zinc finger of Rabphilin-3A, which is closely related to the FYVE domain (a zinc-containing membrane-binding motif), was shown to penetrate the lipid membrane which might result in the optimal conformation for coupling to G-proteins (61).

PA has also been shown to interact with C-Raf (19). The interaction sites for PA have been localized within the C-terminal part of the C-Raf kinase. Deletion mutagenesis of C-Raf revealed that the PA-binding sites are positioned between residues 389 and 423 (19). This region contains a positively charged tetrapeptide sequence RKTR (residues 398–401), which seems to be involved in an initial electrostatic interaction with PA. Additional interactions such as association with the hydrophobic segment ILLFM (residues 405–409) might contribute to the PA binding of C-Raf, whereas disruption of Ras-Raf interaction by C-Raf mutant (R89L) did not affect agonist-dependent translocation (62). These results are

| Binding of C-Raf to | $k_+ \; s^{-1}$ | $K_d \; \text{nm}$ |
|---------------------|----------------|------------------|
| PA                  | $16.8 \pm 0.12 \times 10^4$ | $3.7 \pm 0.20 \times 10^4$ |
| PS                  | $9.2 \pm 0.20 \times 10^4$ | $1.08 \pm 0.04 \times 10^{-3}$ |
| Ceramides           | $5.2 \pm 0.49 \times 10^4$ | $2.69 \pm 0.09 \times 10^{-3}$ |

Ras proteins in a GTP-dependent manner, as already observed for C-Raf fragments containing RBD (43, 49, 58, 59), but did not discriminate between farnesylated and non-farnesylated Ras and between H- and K-Ras (Fig. 2). The same was true for the interactions of the isolated RBD region of C-Raf with farnesylated and non-modified H-Ras (data not shown). The binding of Ras to C-Raf in the lipid vesicle environment was also GTP-dependent (Figs. 3 and 4), but with significantly lower binding efficacy (10–20% compared with 60–70% of coupling efficacy measured in solution). A plausible explanation for this finding might be the fact that lipids (preferentially PS and PA) bind simultaneously with Ras to Raf and compete for the same or neighboring domains, thus reducing the apparent Ras-Raf binding affinity. As shown in Fig. 4, even in the absence of Ras, C-Raf shows high binding tendency for the liposomes consisting of PC, PE, and SM. Using vesicles containing additionally PS and PA, thus mimicking the lipid composition of the inner side of native plasma membrane, C-Raf recruitment by Ras was not detectable, since almost 70% of Raf was bound to lipids in the absence of Ras. These results suggested strongly that Raf kinases might translocate to membranes in the absence of Ras and that Raf possesses a high tendency to associate with lipid environment.

Interactions of Ras Proteins with Raf Kinases—The binding of processed Ras-GTP to C-Raf occurs through two relatively independent interactions (8). Whereas the coupling mechanism of Ras to Raf-RBD are understood in great detail, numerous reports have provided conflicting data with regard to the interactions of Raf-CRD with Ras. Initial studies (49, 58) demonstrated a considerably decreased interaction of Ras with the N-terminal part of CRD when a zinc binding cysteine at position 168 was mutated to serine. These findings have been further confirmed with full-length C-Raf expressed in mammalian cells (10, 11) showing that Raf-CRD is not required for membrane recruitment by Ras but is critical for Raf activation.

In the study presented here we compared the Ras-Raf binding properties in solution and in lipid environment. The full-length C-Raf kinase tested in solution associated with purified lipid vesicles and that Raf translocation to membranes may occur by direct association with phospholipids, lipid second messengers, and cholesterol. Specific interactions have been observed with lipid vesicles containing PS, PA, or ceramides, even in the absence of activated Ras proteins, but the most effective Raf associations with liposomes were observed with cholesterol under the conditions where lipid raft formation is favored. In all instances, lipid association of Raf was accompanied by inhibition of kinase activity.

**Specific Associations of B- and C-Raf Kinase with PS, PA, and Ceramides**

- Evidence that fragments of Raf proteins, particularly C-Raf-CRD, interact with phospholipids have been presented (15, 16).
- The amino acids in C-Raf-CRD interacting with PS have been mapped as Arg-143, Lys-144, and Lys-148, since substitution of all three basic residues to alanine resulted in a C-Raf variant that failed to interact with PS. Additionally, further interactions between Raf and membranes might occur by insertions of hydrophobic residues of C-Raf-CRD into the lipid bilayer. A similar mechanism for membrane attachment has been proposed for Rabphilin-3A, which is an effector of the Ras-related small GTPase Rab3A (17, 60).
- Based on x-ray crystal structure, the zinc finger of Rabphilin-3A, which is closely related to the FYVE domain (a zinc-containing membrane-binding motif), was shown to penetrate the lipid membrane which might result in the optimal conformation for coupling to G-proteins (61).
- PA has also been shown to interact with C-Raf (19).
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- This region contains a positively charged tetrapeptide sequence RKTR (residues 398–401), which seems to be involved in an initial electrostatic interaction with PA.
- Additional interactions such as association with the hydrophobic segment ILLFM (residues 405–409) might contribute to the PA binding of C-Raf, whereas disruption of Ras-Raf interaction by C-Raf mutant (R89L) did not affect agonist-dependent translocation (62).
in agreement with our findings demonstrating that both B- and C-Raf interact with PA, and support a model in which agonist-induced C-Raf recruitment to the membranes could be mediated solely by lipids such as PA and PS. With regard to Raf localization and linkage to other signaling pathways, several observations were reported recently that support the view that Raf might be associated also with intracellular vesicles and activated by alternative mechanisms. In the course of the activation and internalization of G-protein-coupled receptors, C-Raf was found to be complexed with β-arrestin and to be attached to the endosomal vesicles via clathrin-coated pits (63, 64).

Phosphatidic acid and ceramide are considered to act as intracellular lipid second messengers. Interactions of ceramides with C-Raf kinase have been described (20, 21). Our results obtained with purified Raf kinases reconstituted into phospholipid vesicles enriched with ceramides support the findings that ceramides bind to Raf kinases. But contrary to results of Müller et al. (21), we observed that ceramides bind to the regulatory domain of Raf (presumably to CRD) since the BXB form of C-Raf, which corresponds to the catalytic domain, did not exhibit any binding to ceramides (Fig. 5). Taken together, our results with respect to lipid binding performed with purified and functional full-length C-Raf kinases using in vitro reconstitution assay are consistent with observations previously reported (15, 16, 19). We extended these investigations to include B-Raf kinase, an active mutant of C-Raf (C-Raf-Y340D/Y341D) and the BXB form of C-Raf. We show that the binding
Regulation of B- and C-Raf Kinases

specificities of B-Raf and C-Raf for lipids investigated in this study are similar, with the exception that B-Raf also possesses binding affinities for PI. This behavior might be explained by the amino acid composition of the corresponding domains proposed to participate in the Raf binding to PS and PA. Alignment of the PA binding segment of the human C-Raf to that of B-Raf reveals identical amino acid sequences. Similarly, two proposed basic PS-binding residues Arg-143 and Lys-144 are present in both C- and B-Raf. In addition to data presented in Fig. 5 performed by a lipid binding assay, we evaluated kinetic parameters and affinity constants for Raf binding to PS, PA, and ceramides using biosensor technology. According to these data the interactions of C-Raf with PS, PA, and ceramides were shown to be specific with apparent affinity constants in the range of 0.5–50 nM (Table I). In the meantime, the applications of L1 sensor chips for capturing of intact vesicles became widespread: direct interactions between drugs and liposomes (65) and measurements of protein-protein associations in the presence of liposomes (22) have been described. A quantitative approach for the interactions of FYVE zinc finger domains with phosphatidylinositol 3-phosphate has been reported by Gauillard et al. (66). Interestingly, they measured also slow dissociation rates and high affinity binding between analyte and liposomes with a $K_D$ value of 45 nM comparable with our findings.

Interactions of Raf with Cholesterol and Lipid Rafts—Lipid Rafts represent liquid-ordered microdomains distributed into the plasma membrane whose lipid composition differs from the rest of the membrane. The main function of such microdomains would be to concentrate particular signal transduction components into small regions, thus allowing more efficient communication between the outer and inner leaflet of plasma membrane. The high cholesterol levels in rafts enable the tight packing of sphingolipid molecules by occupying the spaces between the saturated fatty acid chains of the sphingolipids. Since rafts could become easily aggregated, detergent-resistant membrane preparations contain not only individual rafts but also clustered rafts and caveolae. Caveolae, exhibiting morphologically defined cell surface invaginations, are supposed to represent a subtype of rafts containing caveolin, a scaffold- and cholesterol-binding structural protein (25, 27, 34, 67). The list of signaling proteins apparently associated with caveolin has increased and includes, among others, Src family kinases, heterotrimeric G-proteins, small G-proteins, adenylyl cyclase, eNOS, nNOS, PKA, PKCa, MEK, ERK, and some receptor proteins (25, 27, 34). Direct interactions of caveolin with signaling molecules led to their inactivation (68). Interactions of caveolin with Raf kinases have not been observed so far. However, association of C-Raf with isolated caveolae/raft fractions prepared by sucrose gradient centrifugation have been reported (35, 38). While Mineo et al. (38) found that C-Raf was accumulated in caveolae/raft fractions after epidermal growth factor stimulation (and Ras activation), Prior et al. (35) located Raf (in the presence of active Ras) to non-raft fractions. In the same article, Prior et al. (35) observed that only non-active H-Ras (Ras-GDP) remains associated with raft/caveolae fractions and that K-Ras, contrary to H-Ras was found to be localized almost completely in non-raft fractions. However, very recently Kranenburg et al. (36) reported, in contrast to Prior et al. (35), that K-Ras co-localized largely with caveolin, whereas N-Ras (which exhibits the same acylation pattern as H-Ras) co-localized to both caveolar and non-caveolar regions of plasma membrane. This discrepancy might be explained by the fact that different cell lines were used.

Having in mind that in vitro isolated raft preparations represent aggregated material, including caveolae and sometimes rafts from subcellular compartments, we decided to investigate Raf interactions with rafts under well defined conditions using in vitro experiments. We prepared liposomes with fixed lipid compositions and monitored Raf association with cholesterol and possibly raft structures (Fig. 9). Recently, the formation and visualization of rafts in model membranes has been described (55, 56). It was shown that lipid mixtures consisting of cholesterol, SM, and GPLs in the proportion of 1:1:1 could form unilamellar vesicles exhibiting properties expected for raft microdomains, as proved by fluorescent labeled phospholipid analogues (55, 56). To monitor Raf association with cholesterol (and rafts), we used lipid vesicle preparations with a defined diameter of 200 nm prepared by extrusion method (47). The lipid compositions and proportions were similar to those described by Dietrich et al. (55, 56) and are indicated in Fig. 9. By using increasing amounts of cholesterol we demonstrate here that Raf associates with cholesterol. Two to 3-fold increase over basal association have been observed by using both column binding assay and biosensor technology. A dramatic increase in Raf binding was observed only in the case that both SM and cholesterol amounts were elevated (Fig. 9C). Since the proportions of lipids were similar to those described by Dietrich et al. (55, 56), we assume that formation of raft microdomains is responsible for this dramatic increase of Raf association with liposomes. Additionally, as shown in Fig. 9 we found that ceramides clearly influence the raft structure and formation and that C-Raf binding to ceramides increased in the presence of cholesterol. This observation is in accordance with data recently published by Xu et al. (69) showing that small amounts of ceramides stabilize raft formation. Since caveolin is not present in our experiments we suggest that Raf association with rafts could take place outside of caveolae. Furthermore,
we propose (see also Fig. 10) that Raf association with lipid rafts occurs by interaction with both ceramides and cholesterol. A limitation of this proposal stems from the lack of knowledge about raft structure. For example, it is currently unknown to what degree the inner and outer leaflet of raft microdomains might differ in lipid composition (26). Production of ceramides occurs metabolically from SM, which is highly enriched in rafts. Therefore it is possible that ceramide synthesis regulates the Raf translocation and further activation process. Preliminary experiments that involve addition of SMase to SM-containing liposomes support this possibility (data not shown). The interactions of Raf with PS and PA probably take place in non-raft regions, since these regions represent GPl-rich domains. Our next effort will focus on investigating other signaling molecules such as Ras, heterotrimeric G-proteins, MEK/ERK, and Src kinases (in the presence and absence of Raf) with respect to their interactions with rafts and to delineate conclusively the constellation of components sufficient for a complete in vitro re-activation of Raf kinases.

Conclusions for the Regulation of Raf Activation-Inactivation Cycle—The currently accepted model for Raf translocation to membranes is based on the observation of Ras-dependent recruitment of Raf induced by external stimuli or constitutive activation by oncogenic mutations (1, 70). Since experiments demonstrating Ras-Raf binding at the plasma membrane generally involved overexpression of Ras and/or Raf, it has to be considered that unphysiologically high levels of Ras in the plasma membranes may be responsible for the apparent Raf translocation. According to data presented here and by others (19, 62), the direct and Ras-independent Raf association with membranes should be considered as the basal Raf translocation pathway. Fig. 10 depicts a model of a Raf activation-inactivation cycle that is consistent with our data. In this model we propose Raf to be targeted to the plasma membrane primarily by its association with cholesterol and ceramides (raft microdomains). The activation process utilizes membrane-prebound Raf that exhibits low basal activity due to the lipid interactions and loss of 14-3-3 proteins (see Figs. 5 and 8). The dissociation of 14-3-3 may facilitate the dephosphorylation of serine at the position Ser-259 (73). Removal of the phosphate at this position by specific phosphatases may be a prerequisite for dissociation of 14-3-3 protein displacement as shown in Figs. 5E and 8 may be the major route in which cells down-regulate B-Raf kinase in quiescent cells.

In summary, we document in this article that interactions of Raf kinases with lipids (i.e. lipid microdomains) are more complex than originally supposed. The inactivation of Raf kinases and 14-3-3 protein displacement are the direct consequences of Raf-lipid interactions. Whether raft domains are also the site where Raf re-activation takes place or whether Raf has to be translocated to other domains in the plasma membrane in the course of activation remains to be elucidated.

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Associations of B- and C-Raf with Cholesterol, Phosphatidylserine, and Lipid Second Messengers: PREFERENTIAL BINDING OF Raf TO ARTIFICIAL LIPID RAFTS

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