B- and C-RAF Display Essential Differences in Their Binding to Ras

THE ISOTYPE-SPECIFIC N TERMINUS OF B-RAF FACILITATES RAS BINDING*□

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Recruitment of RAF kinases to the plasma membrane was initially proposed to be mediated by Ras proteins via interaction with the RAF Ras binding domain (RBD). Data reporting that RAF kinases possess high affinities for particular membrane lipids support a new model in which Ras-RAF interactions may be spatially restricted to the plane of the membrane. Although the coupling features of Ras binding to the isolated RAF RBD were investigated in great detail, little is known about the interactions of the processed Ras with the functional and full-length RAF kinases. Here we present a quantitative analysis of the binding properties of farnesylated and nonfarnesylated H-Ras to both full-length B- and C-RAF in the presence and absence of lipid environment. Although isolated RBD fragments associate with high affinity to both farnesylated and nonfarnesylated H-Ras, the full-length RAF kinases revealed fundamental differences with respect to Ras binding. In contrast to C-RAF that requires farnesylated H-Ras, cytosolic B-RAF associates effectively and with significantly higher affinity with both farnesylated and nonfarnesylated H-Ras. To investigate the potential farnesyl binding site(s) we prepared several N-terminal fragments of C-RAF and found that in the presence of cysteine-rich domain only the farnesylated form of H-Ras binds with high association rates. The extreme N terminus of B-RAF turned out to be responsible for the facilitation of lipid independent Ras binding to B-RAF, since truncation of this region resulted in a protein that changed its kinase properties and resembles C-RAF. In vivo studies using PC12 and COS7 cells support in vitro results. Co-localization measurements using labeled Ras and RAF documented essential differences between B- and C-RAF with respect to association with Ras. Taken together, these data suggest that the activation of B-RAF, in contrast to C-RAF, may take place both at the plasma membrane and in the cytosolic environment.

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External stimuli, such as growth factors or mitogen treatment of the cells, activate RAF kinases via small GTP-binding proteins of the Ras family. These pathways mediate cellular events, such as proliferation, differentiation, and transformation (1, 2). The members of the RAF kinase family, A-, B-, and C-RAF, share three highly conserved regions: CR1, CR2, and CR3. The CR3 region represents the catalytic domain, whereas CR1 contains a Ras binding domain (RBD)4 and a zinc binding domain also called the cysteine-rich domain (CRD). Cytosolic C-RAF exists as a multiprotein complex, including heat shock proteins, 14-3-3 proteins, and kinase suppressor of Ras (3). Upon stimulation of cell surface receptors, C-RAF undergoes a series of activation events that are initiated by Ras proteins, including phosphorylation of serine 338 and tyrosines 340/341 (4–6). The degree of kinase activity of A-, B-, and C-RAF differs considerably. Although A- and C-RAF exhibit moderate levels of basal kinase activities, B-RAF is characterized by extraordinarily high values. These differences have been attributed to the constellation of the acidic residues in the N-region (Y448D and Y449D) of the kinase domain.

The Ras family of GTP-hydrolyzing oncoproteins is part of a large superfamily of more than 150 evolutionarily conserved proteins related to Ras. About 35 members constitute the Ras subfamily, in which H-, N-, and K-Ras represent the best studied members (7, 8). Most of the Ras proteins become posttranslationally modified by C-terminal farnesylation or geranylgeranylation. As a result, Ras is translocated to the inner surface of the plasma membrane. However, recent studies suggested that Ras signaling may not be restricted to the plasma membrane but could also occur at the Golgi apparatus and other endomembranes (9, 10). Moreover, a number of small G-proteins have been described (7, 8) that are not post-translationally lipidated and that can transform cells in their constitutively active form and induce differentiation events, such as neurite outgrowth (11).

GTP-bound Ras associates with a large number of effectors, such as RAF kinases, phosphatidylinositol 3-kinase, and Raf-
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GDS. Although Ras proteins play a crucial role in the activation of C-RAF, the exact mechanism of Ras-RAF coupling is not completely understood. The RBD of C-RAF comprises the residues 51–131 and binds directly to the switch-I region of active Ras-GTP (12). However, in addition to the RBD, the CRD domain of C-RAF encompassing residues 139–184 appears to play an important role in Ras-RAF coupling and activation of RAF. Whereas the Ras-RBD interaction is understood in great detail, a number of reports have provided conflicting data regarding the role of CRD. Initial studies (13, 14) demonstrated a considerably decreased interaction of Ras with the N-terminal part of C-RAF when some of the zinc binding cysteines were mutated to serine. These findings have been confirmed with full-length C-RAF expressed in mammalian cells (15, 16). The possibility that the farnesyl residue of Ras may interact directly with the hydrophobic surface of CRD has been proposed. To corroborate this hypothesis, the interactions of farnesylated and nonfarnesylated H-Ras with the isolated C-RAF-CRD have been investigated (17, 18). Using fluorescence-based binding assays and NMR spectroscopy, the authors observed that only farnesylated Ras binds to CRD. However, as previously demonstrated (19), the Ras-CRD interactions were essentially independent of the guanine nucleotide state of Ras. The relatively weak affinity constant for binding of farnesylated Ras to CRD ($K_D$ of about 20 nM) has been explained by the fact that the tight binding of Ras-GTP to RBD compensates in vivo for this weak interaction. Besides the ability to interact with processed Ras, CRD was reported to possess binding sites for 14-3-3 proteins and phosphatidylinerine (20, 21). Thus, CRD reveals a multifunctional role with respect to the regulation of C-RAF activation. These findings supported a dual role for Ras: (i) tight coupling of Ras-GTP to C-RAF RBD with high affinity constants and (ii) weaker coupling of farnesyl-Ras to the CRD, a process that seems to be necessary for induction of C-RAF activation. On the other hand, lipidation is also indispensable for association of Ras with membranes, as illustrated by the use of farnesyl-sulfonate-deficient Ras mutants (22, 23). Thus, the palmitoyl residues are of importance for physical anchoring of Ras to membranes, as demonstrated by Bader et al. (24), who showed that the stability of Ras insertion into artificial membranes correlates with the degree of hydrophobic modifications. Additionally, Rocks et al. (10) reported that the de-/repalmitoylation of H-Ras plays an important role in the subcellular distribution, driving rapid exchange of Ras between plasma membrane and Golgi apparatus.

In contrast to C-RAF, where binding studies with the N-terminal part of C-RAF, including RBD and CRD, have been reported (14), little is known about the binding of B-RAF to Ras. It has been proposed that B-RAF may couple to Rap1 and Rit1 as well (11, 25). However, no quantitative binding data are available so far. Since the discovery of activating B-RAF mutations in human cancer (26), RAF research has focused strongly on B-RAF and its activation-deactivation pathways. Among more than 45 mutations in B-RAF described, the substitution of a valine residue at position 600 for glutamic acid (V600E) is the most frequently occurring alteration (27). It has been reported recently that individuals with CFC (cardio-facio-cutaneous) syndrome bear either K-Ras or a variety of B-RAF mutations (28, 29). Importantly, for the first time, B-RAF mutations were identified that are normally involved in the membrane association (i.e. mutations were found in the CRD region (A246P and Q257R) and phosphatidic acid (PA) binding domain (K499E)). We have previously demonstrated that lipid binding is crucial for the reduction of the high basal B-RAF activity in unstimulated cells (30), indicating that the impaired lipid binding might lead to a permanent active B-RAF kinase. Interestingly, all of the lipid-deficient mutants mentioned above revealed significantly higher kinase activity compared with B-RAF wild type.

To compare in a quantitative manner the interactions of Ras with isolated RAF RBDs and full-length B- and C-RAF, we applied in the current study biosensor technology based on surface plasmon resonance, which allows monitoring of biomolecular interactions in real time. We report here that, contrary to the isolated RBD fragment, the full-length C-RAF associates preferentially with farnesylated Ras. The significantly weaker interaction of C-RAF with the GDP form of farnesylated Ras may account for the proposed precoupled state between Ras and RAF that may exist prior to the cell stimulation by external agents (30). B-RAF, on the other hand, exhibits profoundly different binding properties with regard to Ras binding when compared with C-RAF. We found that B-RAF binds with similar affinities both farnesylated and nonfarnesylated H-Ras in solution (mimicking the cytosolic environment). This observation implicates that B-RAF does not necessarily need recruitment to the plasma membrane in order to associate with Ras. Furthermore, our data demonstrate that the higher accessibility of Ras to B-RAF is caused by its extended N terminus, which probably keeps B-RAF in an open conformation.

EXPERIMENTAL PROCEDURES

Materials—Benzamidine, leupeptin, aprotinin, and phenylmethylsulfonyl fluoride were obtained from Sigma. Nonidet P-40, GTP, GTP$\gamma$S, GDP, and GDP$S$ were from Roche Applied Science. Glutathione-Sepharose was purchased from Amersham Biosciences, and Ni$^{2+}$-nitrotriacetic acid-agarose was from Qiagen. The phospholipids and sphingomyelin were from Avanti Polar Lipids, LLC. The phospholipids were identified that are normally involved in the membrane association (i.e. mutations were found in the CRD region (A246P and Q257R) and phosphatidic acid (PA) binding domain (K499E)). We have previously demonstrated that lipid binding is crucial for the reduction of the high basal B-RAF activity in unstimulated cells (30), indicating that the impaired lipid binding might lead to a permanent active B-RAF kinase. Interestingly, all of the lipid-deficient mutants mentioned above revealed significantly higher kinase activity compared with B-RAF wild type.

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Infection of SF9 Cells, Purification of Full-length RAF Kinases, SDS-PAGE, and Western Blot Analysis—For the production of recombinant B- and C-RAF kinases SF9 cells were infected with the desired baculoviruses at a multiplicity of infection of 5 and incubated for 48 h at 27 °C. The cells were then washed with phosphate-buffered saline (PBS) buffer and pelleted for 5 min at 500 × g. Cell pellets (2 × 10$^8$ cells) were lysed in 10 ml of Nonidet P-40 lysis buffer containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM sodium pyrophosphate, 25 mM β-glycero-
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HEK293 cells were cultivated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (heat-inactivated at 56 °C for 45 min), 2 mM l-glutamine, and 100 units/ml penicillin/streptomycin at 37 °C in humidified air with 5% CO2. HEK293 cells were seeded at 7.5 × 105 cells/well on a 6-well plate and grown for 24 h before transfecting them with expression plasmids using either the calcium phosphate method or the liposome-mediated transfection method (Lipofectamine; Invitrogen). 16 h post-transfection cells were washed twice with PBS to remove serum traces and cultivated for the indicated time in medium supplemented with 0.3% serum to keep the activity of endogenous kinases low. Cells were washed once in PBS, and equal amounts of cells were lysed either by direct addition of Laemmli buffer or by Nonidet P-40-containing lysis buffer (10 mM Heps, pH 7.5, 142.5 mM KCl, 5 mM β-glycerophosphate, 25 mM NaF, 10% glycerol, 0.75% Nonidet P-40, and a mixture of standard protease inhibitors (Nonidet P-40 buffer) 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 containing GST-tagged RAF kinases were incubated with 0.5 ml of glutathione-Sepharose beads for 2 h at 4 °C with rotation. After incubation, the beads were washed three times with Nonidet P-40 buffer containing only 0.2% Nonidet P-40. RAF kinases bound to the beads were eluted with 0.5 ml of 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 25 mM β-glycerophosphate, 25 mM NaF, 10% glycerol, 0.2% Nonidet P-40, and 20 mM glutathione. The purity of RAF kinases was documented by SDS-polyacrylamide gel electrophoresis (10% gels) and staining with Coomassie Blue (Fig. 2). For Western blot analysis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) and probed with antibodies specific for B- or C-RAF and phospho-ERK. After washing, the membranes were incubated with specific secondary horseradish peroxidase-conjugated antibodies and detected by ECL (Amersham Biosciences). The degree of C-RAF phosphorylation in positions serine 338 and tyrosine 340/341 was detected using phosphospecific antibodies directed against these residues as indicated in Fig. 2.

Cell Culture, Transfection, and Immunoprecipitation—

Full-length B- and C-RAF proteins and their fragments were purified as GST-tagged proteins and immobilized on the biosensor chip that was coated with anti-GST antibody. To monitor association of Ras to RAF, the purified and GTP-loaded farnesylated (Ras-farn) and nonfarnesylated (Ras) proteins and immobilized on the biosensor chip that was coated with anti-GST antibodies and detected by ECL (Amersham Biosciences). The degree of C-RAF phosphorylation in positions serine 338 and tyrosine 340/341 was detected using phosphospecific antibodies directed against these residues as indicated in Fig. 2.
heterodimerization between B- and C-RAF was performed as described (5). Briefly, the HEK293 cells were transfected with plasmids encoding HA-tagged C-RAF and additionally either B-RAF WT or B-RAFΔN98 mutant in the presence of H-Ras12V. The cells were lysed with lysis buffer, and the immunoprecipitation was performed with an anti-HA antibody (clone 3F10; Roche Applied Science). The washed precipitates were boiled for 5 min at 100 °C and applied to SDS-PAGE. The proteins of interest were visualized by Western blotting using monoclonal anti-Ras antibody (R-02120; Transduction Laboratories) and antibodies against B- and C-RAF (Santa Cruz Biotechnology).

RAF and Ras Expression Plasmids, Nucleotide Loading, and Farnesylation of Ras—Expression and purification of H-Ras and H-Ras12V were performed as described before (32). Isoprenylation of full-length H-Ras protein was carried out with Saccharomyces cerevisiae protein farnesyltransferase (33). Briefly, 500 nmol of H-Ras was incubated with 20 nmol of farnesyltransferase and 1 mol of farnesyl pyrophosphate in 30 mM Tris-HCl, pH 7.4, 50 mM NaCl, 20 μM ZnCl₂, 5 mM octyl glucoside, and 2 mM dithiothreitol in a total volume of 5 ml for 3 h at 30 °C. Farnesylated product was separated from nonfarnesylated H-Ras by extraction with Triton X-114 (34), followed by removal of the detergent with ion exchange chromatography (DEAE-Sepharose column). Integrity of the product was verified by electrospray ionization mass spectroscopy. The Ras proteins were loaded with nucleotides as described (30). The samples were stored at −20 °C in the presence of nucleotides and MgCl₂, since removal of these reagents led to inactivation of Ras-GTP upon prolonged storage, an observation that is in accordance with findings already reported (35). Plasmids for H-RasG12V and H-RasG12V-C186S have been described elsewhere (36). The GTPase sequences were introduced into pDsRed1-C1 (Clontech) by PCR cloning for expression of GTPase proteins in fusion with amino-terminally located DsRed1 (23). The RBDs of RAF kinases and different N-terminal fragments were prepared as GST fusion proteins and purified from Escherichia coli. For that purpose, RAF deletion mutants were PCR-amplified with primer overhangs containing the restriction sites for cloning. PCR products were cleaved with BamHI + NotI restriction enzymes and cloned into GST fusion vector pGEX-4T-3 cleaved with the same enzymes. The proteins were expressed in E. coli DH5α

FIGURE 2. Expression and purification of Ras proteins, B- and C-RAF kinases, and their fragments. The RAF kinases and their truncation fragments used for Ras/RAF binding studies are schematically illustrated in A. The full-length C-RAF kinases and B-RAFΔN98 were purified from Sf9 insect cells as GST fusion proteins. The highly activated C-RAF-R/L was expressed in the presence of Ras12V and Lck. Truncation fragments of B- and C-RAF were purified from E. coli as GST fusion proteins. Ras proteins were purified and lipidated as described under “Experimental Procedures.” The purity of RAF and Ras preparations is documented by SDS-PAGE and Coomassie Blue staining. The C-RAF proteins were associated with HSP70 to a minor degree. The C-RAF phosphorylation in positions serine 338 and tyrosine 340/341 was analyzed by phosphospecific antibodies as indicated. The kinase activity of the RAF preparations was assayed in the presence of recombinant MEK and ERK and monitored by phospho-ERK antibody (B).
cells after induction with isopropyl 1-thio-β-D-galactopyranoside at 25 °C overnight. The cell lysates were incubated with glutathione-Sepharose, and after washing procedures the GST-tagged proteins were eluted with 20 mM glutathione in 50 mM Tris-HCl buffer, pH 8, and 10 mM β-mercaptoethanol.

**Kinase Activity Measurements**—Kinase assays with RAF samples were performed using recombinant MEK and ERK-2 as substrates in 25 mM Hepes, pH 7.6, 150 mM NaCl, 25 mM β-glycerophosphate, 10 mM MgCl₂, 1 mM dithiothreitol, and 1 mM sodium vanadate buffer (50-µl final volume). Following the addition of RAF- and Ras-containing samples and 1 mM ATP, the reaction mixtures were incubated for 30 min at 30 °C. The incubation was terminated by the addition of Laemmli sample buffer, and the proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The extent of ERK phosphorylation was determined with anti-phospho-ERK antibodies.

**Fluorescence Microscopy and Quantification of Neurite Outgrowth**—To assess co-localization between RAF kinases and H-Ras and their differentiation potential, we used PC12 and COS7 cells. PC12 cells were cultured in Dulbecco’s modified Eagle’s medium, 10% horse serum, and 5% fetal calf serum. For Ras/RAF localization studies, cells were transiently transfected with 0.9 µg of expression plasmid for pEGFP, pDsRed1-HRas-G12V, pDsRed1-HRas-G12V/C186S, pEGFP-B-RAF, pEGFP-C-RAF using Lipofectamine 2000. Transfection was performed according to the protocol for 24-well plates as provided by the manufacturer (Invitrogen). Cells were kept over-night in the transfection solution and then trypsinized and plated on 4-well plates (5000 cells/cm²). Cells were then grown another 48 h before they were fixed with 4% paraformaldehyde in PBS, pH 7.4. Cells were then washed three times with Tris-buffered saline (25 mM Tris-HCl, pH 7.4, 0.8% NaCl, 0.2% KCl) and 0.05% Tween 20, the last wash being performed for 10 min in the presence of 10 µM 4’,6-diamidino-2-phenylindole. The cells were washed another two times with Tris-buffered saline, covered with Mowiol in 50% glycerol/PBS, and observed under a Leica confocal microscope (TCS, Leica, Heidelberg, Germany). Fluorescent signals were analyzed using Leica confocal image software. Single estimations were pooled, and the results were expressed as mean and S.E. Statistical significance of recorded differences was assessed by analysis of variance followed by Bonferroni’s test using Prism (GraphPad, San Diego, CA). COS7 cells were transfected at subconfluence with Lipofectamine according to the manufacturer’s instructions. 48 h after transfection, the cells were prepared for confocal microscopy as described previously.

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![Figure 3. Quantitative biosensor analysis of the interactions between H-Ras and N-terminal fragments of C-RAF (C4, C5, and C-RAF-ΔBXB) (A and B) and B-RAF-ΔBXB (C). N-terminal fragments of RAF (see also Fig. 2) were purified from E. coli as GST fusion proteins. To monitor the association-dissociation curves for the Ras/RAF interactions, first the truncated RAF proteins were captured by the immobilized anti-GST antibodies. Next, the farnesylated and nonfarnesylated H-Ras samples were applied at the indicated concentrations. In A, only the results obtained using 800 nM Ras are shown. These measurements were repeated two times.](image-url)
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(23). For quantification of neurite outgrowth, pictures were taken from confocal (Leica) or fluorescent microscopic analysis (Zeiss, Axiophot). Neurite length was measured using the Scion Image software. At least three independent experiments were performed for data collection.

Biosensor Measurements—The biosensor measurements were carried out either on the BIAcore-X or BIAcore-J system (Biacore AB, Uppsala, Sweden) at 25 °C. To measure Ras-RAF interactions in the absence of liposomes, the biosensor chip CM5 was loaded with anti-GST antibody using covalent derivatization according to the manufacturer’s instructions. Purified and GST-tagged proteins were immobilized in Biosensor buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, and 0.01% Nonidet P-40) at a flow rate of 10 ml/min, which resulted in a deposition of approximately 1100–1200 response units (RU) in the case of full-length RAF kinases. Next, the purified analytes (farnesylated and nonfarnesylated H-Ras proteins) were injected at the indicated concentrations. The values for nonspecific bindings measured in the reference cell were subtracted. Large unilamellar vesicles were prepared using a LiposoFast extrusion apparatus (Avestin Inc., Canada) as described (30). To measure interactions between RAF and Ras at the surface of artificial membranes, Pioneer L1 sensor chips (Biacore AB, Uppsala, Sweden) were used. For that purpose, the surface of the sensor chips was first cleaned with 20 mM CHAPS followed by injection of PA-containing liposomes (0.4 mM lipid concentration) at a flow rate of 10 μl/min in 10 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, and 0.1 mM dithiothreitol, which resulted in a deposition of ~4000 RU in both flow cells. To measure the interactions of B- and C-RAF with farnesylated and nonfarnesylated H-Ras, purified RAF was injected only in the first flow cell (Fc1) and
captured by liposomes at a flow rate of 10 μl/min. After binding of RAS to liposomes, Ras was applied into both flow cells at the same flow rate. The nonspecific binding of Ras to liposomes was subtracted (Fc1 − Fc2 value). At the end of the binding assay, the sensor chip surface was regenerated by injection of 20 mM CHAPS.

The evaluation of kinetic parameters was performed by nonlinear fitting of binding data using the Biacore 2.1 analysis software. The apparent association (k_a) and dissociation rates constant (k_d) were evaluated from the differential binding curves (Fc2 − Fc1) assuming an A + B = AB association type for the protein-protein interaction. The dissociation constant K_D was calculated from the equation, K_D = k_d/k_a.

RESULTS

The Ras-RAS binding data so far available have been obtained exclusively by using RAS fragments, mostly RAS RBD, and nonfarnesylated Ras (37, 38). Therefore, it was of interest to investigate whether full-length RAS kinases display the same binding properties as the isolated RBD. The kinetic parameters and affinity constants for the Ras-RBD interactions have been derived previously from experiments performed in solution. We present here data obtained with a biosensor approach, which represents a biphasic system and thus resembles more the native conditions at the plasma membrane. Additionally, we examined Ras-RAS interactions in the presence of artificial membranes that were immobilized to the L1 biosensor chip.

Isolated RAS RBDs Do Not Discriminate between Farnesylated and Nonfarnesylated H-RAS—We examined first Ras binding to isolated RBD fragments. For that purpose, GST-RBD fragments of C-, B-, and A-RAS were captured by a CM5 biosensor chip coated with anti-GST antibody, and purified Ras samples were injected. As shown in Fig. 1, rapid associations and relatively slow dissociations were monitored for this interaction, resulting in K_D values between 10 and 80 nM (Table 1). No significant differences were measured for farnesylated or nonfarnesylated H-Ras (only the binding curves measured for C-RAS-RBD interactions are shown). These findings are in agreement with results obtained by stopped-flow measurements (31, 37, 38) with the exception that, using the biosensor technique, the B-RAS RBD exhibited the highest affinity for H-Ras (see Table 1). The association of Ras-GDP with C-, B-, or A-RAS RBDs was at the limit of detection using surface plasmon resonance technology consistent with data reported by Sydor et al. (38), who measured a K_D value of 32 μM for such interactions.

Cysteine-rich Domain of C-RAS Is Involved in Ras Binding—It has been shown previously that the CRD region of C-RAS plays an important role in Ras binding and RAS activation (13–16). Furthermore, it has been proposed that the farnesyl residue of Ras may interact directly with the hydrophobic surface of C-RAS-CRD (17, 18). However, experiments performed either with isolated RBD or CRD clearly may not reflect the binding properties of full-length RAS. For example, domains other than the CRD may also contribute to the interaction with the farnesyl residue. Therefore, to investigate the role of CRD in Ras binding in more detail, we prepared the entire regulatory domain of C-RAS (C-RAS ΔCRD) and several fragments derived from this region containing both RBD and CRD (deletion mutants C4 and C5; see Fig. 2, A and B). The binding studies carried out with farnesylated and nonfarnesylated H-Ras and C5, C4, and C-RAS ΔCRD (Fig. 3, A and B) showed unambiguously that, in the presence of CRD, only farnesylated Ras coupled with high affinity to RAS fragments. Furthermore, it is also evident from the binding curves that the rate of association of the nonprocessed Ras decreases with the inclusion of C-terminal sequences in C-RAS constructs with the consequence that full-length C-RAS does not accept nonprocessed Ras at all (Table 1). We omitted here to investigate Ras binding to constructs mutated at the cysteines located in the CRD region, since such alterations could change the conformation of the full-length molecule. To compare the B-RAS regulatory domain with that of C-RAS regarding their ability to associate with Ras, we tested also B-RAS ΔCRD lacking the entire catalytic region (see Fig. 2). As illustrated in Fig. 3C, Ras binding to B-RAS ΔCRD displayed fundamental differences compared with C-RAS ΔCRD; both Ras preparations coupled efficiently and with similar affinity constants (K_D values of ~60 nM) to the N-terminal regulatory part of B-RAS. These results indicate that B-RAS does not require the farnesyl moiety of Ras for effective coupling.

Full-length B- and C-RAS Reveal Essential Differences in Their Binding Properties to Ras—The binding constants for Ras interactions with functional full-length C-RAS have not yet been reported. To investigate these interactions, we purified B- and C-RAS kinases from S9 insect cells as GST fusion proteins. The highly activated form of C-RAS wild type, here designated as C-RAS ΔCRD, was expressed in the presence of H-Ras12V and Lck, a tyrosine kinase belonging to the Src family of kinases. The purity of full-length RAS kinases and Ras preparations is documented in Fig. 2B. Using C-RAS phosphospecific antibodies, we show here in accordance with previously published data (4, 39) that C-RAS ΔCRD is highly phosphorylated in positions Tyr340/341 in contrast to C-RAS WT. Phosphorylation of serine 338 was detected in both C-RAS forms although to a lesser extent in the C-RAS preparations without Ras/Lck activation. The corresponding position in B-RAS (serine 446) was highly phosphorylated, as already described (4). Kinase activity measurements using MEK and ERK as substrates revealed considerably higher activity for C-RAS ΔCRD compared with C-RAS (Fig. 2B). The extent of kinase activity of B-RAS was comparable.

FIGURE 4. Quantitative biosensor analysis of the interactions between farnesylated and nonfarnesylated H-Ras and full-length C-RAS (A and C), and B-RAS (B and D). A and B, purified and GST-fused RAS kinases were captured by immobilized anti-GST antibodies, resulting in a deposition of approximately 1100–1200 RU. Next, the farnesylated or nonfarnesylated H-Ras samples were injected at the indicated concentrations. These experiments were repeated three times. C and D, association of Ras with C- and B-RAS in the presence of artificial membranes. The liposomes consisting of phosphatidylethanolamine (37%), sphingomyelin (13%), phosphatidylcholine (20%), and phosphatidic acid (10%) were immobilized at the L1 sensor chip as described (40). To monitor the interactions of C- and B-RAS with Ras, the purified Ras samples were injected only in the first flow cell (Fc1). After binding of Ras to liposomes (approximately 1000 RU were captured), the farnesylated and nonfarnesylated H-Ras was applied into both flow cells. The non-RAS-specific binding of Ras-farnesyl recorded in the second flow cell (Fc2) was subtracted. These experiments were repeated three times.
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Table 1. Kinetic Parameters for Binding of Raf to Ras-GTP

| Protein | Ras-Protein | $K_d$ (nM) | $k_{on}$ ($10^{10}$ M$^{-1}$ s$^{-1}$) | $k_{off}$ (s$^{-1}$) |
|---------|-------------|------------|---------------------------------------|------------------|
| B-RAF   | GDP-Ras     | 46         | 28                                    | 0.07             |
| A-RAF   | GDP-Ras     | 22         | 37                                    | 0.07             |
| C-RAF   | GDP-Ras     | 62         | 23                                    | 0.07             |

with C-RAF-R/L as previously reported (30). Surprisingly, we did not detect binding of nonfarnesylated H-Ras-GTP to either C-RAF preparation. In contrast, application of farnesylated H-Ras-GTP resulted in nucleotide- and concentration-dependent interaction with C-RAF, yielding $K_d$ values of 0.46 and 0.86 nM for C-RAF and C-RAF-R/L, respectively (Fig. 4A and Table 1). The GDP form of farnesylated H-Ras bound to C-RAF with significantly lower affinity, resulting in $K_d$ values of $\sim 10$ μM (data not shown). Whereas C-RAF associated with modest affinity and with a strong preference for farnesylated H-Ras, B-RAF exhibited fundamentally different binding properties. As demonstrated in Fig. 4B, purified B-RAF coupled with high efficiency to both farnesylated and nonfarnesylated H-Ras. Both Ras proteins bound to B-RAF with significantly higher affinities compared with C-RAF, yielding $K_d$ values in the range between 60 and 80 nM (Table 1). The association of B-RAF with GDP-loaded farnesylated Ras was also recordable, albeit only as a low affinity interaction, as in the case of C-RAF.

To investigate the Ras/RAF interaction at the interface of artificial membranes, we performed surface plasmon resonance binding studies with liposomes immobilized to the biosensor chips. We previously showed that B- and C-RAF associate efficiently with phosphatidylinerine- and PA-containing liposomes (30). Especially, association of RAF with PA-containing liposomes has been reported to be crucial for C-RAF activation (40). Therefore, we investigated Ras/RAF interactions in the presence of liposomes containing typical plasma membrane lipids, such as phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and phosphatidylserine. Additionally, the liposomes were enriched with PA. Surprisingly, in the presence of artificial membranes, both B- and C-RAF kinases activated only farnesylated Ras proteins (Fig. 4, C and D). Under these conditions, relatively fast association ($k_{on} \sim 8 \times 10^{9} M^{-1} s^{-1}$) and dissociation rate constants ($k_{off} \sim 8 \times 10^{-3} s^{-1}$) were measured, yielding $K_d$ values of $\sim 1$ μM. These results indicate that membrane association of RAF renders RBD inaccessible to unprocessed Ras.

The binding data obtained with full-length RAF kinases shown in Fig. 4 raised the question as to the molecular basis for the observed isotype-specific differences between B- and C-RAF with respect to Ras binding. A comparison of the regulatory domains in a sequence alignment shows that both kinases possess regions of high homology, particularly in RBD and CRD (Fig. 5). Therefore, it is unlikely that RBD or CRD are responsible for differences in the Ras binding properties of B- and C-RAF. Isozyme-specific sequences such as the N-terminal extension of B-RAF seem more likely candidates. Hence, we speculated that the first 98 amino acids may determine the Ras binding properties of B-RAF by forcing constitutive exposure of B-RAF RBD, a configuration that requires in the case of C-RAF the interaction of the farnesyl residue with CRD.

The N-terminal Domain of B-RAF Determines Ras Binding and the Level of Kinase Activity—To evaluate whether the N-terminal B-RAF-specific sequences influence Ras binding, we prepared a B-RAF deletion mutant lacking the first 98 residues (B-RAFΔN98). Examination of transfected mammalian cells demonstrated that this B-RAF mutant had indeed acquired kinase properties reminiscent of C-RAF. We performed a series of functional assays using B-RAF and B-RAFΔN98 (Fig. 6) and show that (i) B-RAFΔN98 exhibits much lower basal kinase activity than B-RAF with respect to MEK/ERK phosphorylation, (ii) the -fold stimulation of B-RAFΔN98 by EGF (5-fold) is similar to that of C-RAF (7-fold), and (iii) BAD as a second target of B-RAF was phosphorylated to a much lower degree by B-RAFΔN98 relative to wild type B-RAF (Fig. 6A). These data show clearly that the removal of the extreme N-terminal segment in B-RAF changes completely its kinase properties and resulted in a kinase that is very similar to C-RAF with the exception that B-RAFΔN98 still has higher basal activity presumably due to the presence of phosphomimetic residues Asp448 and Asp449 in the N-region.

Next, we investigated whether the N-terminally truncated B-RAF (B-RAFΔN98) still maintained the ability to form heterodimeric complexes with C-RAF as reported for the full-length B-RAF (5, 41, 42). As shown in Fig. 6B, heterodimer formation between C-RAF and B-RAFΔN98 occurred in HEK293 cells in a Ras-dependent manner and to degrees similar to those reported for full-length B-RAF. Moreover, mutation of serine 621 to alanine in the C-terminal 14-3-3 binding motif of C-RAF considerably reduced the extent of heterodimer formation (data not shown), indicating that 14-3-3 adaptor proteins regulate this process. These observations are not consistent with the data of Terai and Matsuda (43), who reported recently that the N-terminal region of B-RAF mediates heterodimerization with C-RAF.

Finally, to compare Ras association with B-RAF WT and B-RAFΔN98, we performed binding assays using two different approaches. First, we used immunoprecipitated material from mammalian cells transiently transfected with B-RAF WT or

![Sequence alignment of the N-terminal fragments of A-, B- and C-RAF containing RBD and CRD regions.](image-url)

FIGURE 5. Sequence alignment of the N-terminal fragments of A-, B- and C-RAF containing RBD and CRD regions.
B-RAF/H9004N98. The equilibrium binding assays shown in Fig. 7, A and B, reveal that nonfarnesylated Ras associates more efficiently with B-RAF WT, indicating that the N-terminal truncation in B-RAF reduces the accessibility for nonprocessed Ras. The $K_D$ values for these interactions derived from the half-maximal binding of Ras to full-length B-RAF and B-RAFΔN98 are about 80 and 400 nM, respectively. This is in accordance with the results obtained by BIAcore measurements demonstrating that binding of farnesylated and nonfarnesylated Ras to B-RAFΔN98 is more similar but not identical to C-RAF rather than B-RAF binding (Fig. 7C and Table 1). These results may also reflect differences in affinity of the isolated RBDs of the particular RAF kinase (compare $K_D$ values for Ras binding to the isolated RBDs of A-, B-, and C-RAF, Table 1).

On the other hand, the addition of the first 98 amino acids of B-RAF to the N terminus of C-RAF might be expected to result in elevated basal kinase activity. To test this possibility, we prepared a chimeric form of C-RAF as illustrated in Fig. 2A. Only a moderate increase (25%) of B/C-RAF kinase activity was observed relative to C-RAF WT (data not shown).

**B- and C-RAF Co-localize Differentially with Ras in Vivo**—To examine whether B- and C-RAF display different subcellular distributions in mammalian cells as a function of Ras activation and prenylation, we analyzed Ras/RAF co-localization in vivo by confocal microscopy. For that purpose, we used COS7 and PC12 cells transiently transfected with either B- or C-RAF that were fused to GFP. Additionally, H-Ras12V or H-Ras12V mutant lacking the cysteine 186 necessary for the attachment of the farnesyl residue (H-Ras12V/186S) was co-expressed with RAF. For co-localization analysis, both Ras proteins were fused to a red fluorescent protein. Cell imaging revealed a considerable fraction of H-Ras12V associated with the plasma membrane due to prenylation and palmitoylation of Ras. In addition, varying amounts of H-Ras12V were detected at a perinuclear globular structure, which represents the Golgi apparatus (see Figs. 9 and S3), as shown previously (9, 10, 23). Both RAF kinases co-localized with H-Ras12V at the plasma membrane, with C-RAF exhibiting a considerably higher degree of co-localization with farnesylated Ras compared with B-RAF (Figs. 8 and S1). In marked contrast, prenylation-deficient Ras (H-Ras12V/186S) did not associate with plasma membrane and Golgi and was detected predominantly in the cytoplasm.

Importantly, it is evident from the immunofluorescence data that B-RAF, in contrast to C-RAF, becomes homogenously distributed in the presence of farnesyl-deficient Ras mutant (H-Ras12V/186S), indicating that B-RAF interacts in the cytosolic environment with nonlipidated or partially lipidated Ras protein. In addition, we observed that both B- and C-RAF co-localize with H-Ras12V at the Golgi (Fig. S3). However, in the presence of the H-Ras12V/186S variant, only B-RAF was depleted from the Golgi, indicating again that B-RAF may

**FIGURE 6.** Truncation of the N-terminal part of B-RAF changes its kinase properties. The full-length B-RAF and B-RAF lacking the first 98 amino acids (B-RAFΔN98) were expressed in HEK293 cells. A, the kinase activities of the RAF kinases were assayed either in the presence of recombinant MEK and ERK or BAD as substrates. For that purpose, lysates (5 μg of total protein) were used. To monitor changes in kinase activity dependent on cell stimulation, the cells were starved and treated with EGF (100 ng/ml) for 5 min. These experiments were repeated three times. B, the heterodimerization between B- and C-RAF is not impaired by truncation of the B-RAF N terminus. HEK293 cells were transfected with HA-tagged C-RAF and with either B-RAF WT or B-RAFΔN98 in the presence of Ras12V or Ras12V/186S proteins, as indicated. The cells were lysed with Nonidet P-40 buffer, and the RAF dimers were precipitated (IP) using anti-HA antibody. After SDS-PAGE and Western blotting (WB), the degree of heterodimerization was visualized by specific anti-RAF antibodies.
interact with soluble forms of Ras protein (Fig. 8). Thus, these results demonstrate that B-RAF, contrary to C-RAF, associates in vivo to a much lower degree with farnesylated Ras and suggest strongly that B- and C-RAF become activated by different mechanisms. This observation supports our in vitro binding assays (Figs. 4 and 7), demonstrating that B-RAF, in contrast to C-RAF, may also accept partially lipidated Ras proteins.

Are there physiological consequences of this selective binding of nonfarnesylated Ras to B-RAF? To answer this question, we used the special property of PC12 cells to respond with neurite outgrowth to stimulation with Ras and/or RAF proteins. As depicted in Fig. 8B, nonfarnesylated Ras (H-Ras12V/186S) alone was able to induce differentiation. This occurs most likely by interaction with endogenous B-RAF. Co-expression of H-Ras12V/186S with B-RAF strongly increased neurite outgrowth to the level achieved by H-Ras12V; a comparable effect was not seen with C-RAF.

To investigate whether immunofluorescence data correlate with in vivo binding of B- and C-RAF to differentially lipidated Ras proteins, we used Sf9 insect cells. For that purpose, GST-tagged B- or C-RAF was co-expressed with either H-Ras12V or farnesyl-deficient mutant H-Ras12V/186S. Additionally, we investigated the Ras binding properties of the N-terminally truncated B-RAF (B-RAFΔN98). RAF proteins were precipitated from lysates by glutathione-Sepharose, and the amount of associated Ras was detected by Western blot analysis. As illustrated in Fig. S2, C-RAF and B-RAFΔN98 revealed a clear preference for the fully lipidated Ras protein. On the other hand, B-RAF WT associated to a similar extent with both H-Ras12V and H-Ras12V/186S. Thus, the results from Sf9 cells together with immunofluorescence data (Figs. 8 and S1) are in full agreement with our in vitro binding assays.

Reconstitution Experiments with Purified Ras and RAF—Since farnesylation of H-Ras increased considerably the binding efficiency of Ras to C-RAF, we addressed the question whether these interactions are sufficient for direct RAF activation in vitro. For that purpose, C-RAF was incubated with farnesylated Ras-GTP and Ras-GDP samples in the presence of recombinant MEK and ERK. Monitoring ERK phosphorylation, we did not observe significant enhancement of RAF activity in the presence of Ras proteins (data not shown). Apparently, binding of farnesylated Ras to RAF is not sufficient to increase RAF kinase activity. These data support a model for C-RAF activation that includes phosphorylation/dephosphorylation events subsequent to Ras/RAF interaction (1).

DISCUSSION

Quantitative data regarding interactions of (nonfarnesylated) Ras proteins with isolated RBDs have been previously reported (37, 38, 44). In contrast, data using lipidated Ras and full-length RAF are not available so far. The reason for that may lie in the presumed low affinity constants for this interaction. Particularly, it has been assumed that the dissociation of Ras
from C-RAF may be very rapid. Indeed, using the experimental technique described here in the presence of artificial membranes, a significantly faster dissociation of Ras from the full-length C-RAF was observed, indicating a shorter lifetime for this complex compared with the Ras-RBD complex.

Most members of the Ras protein subfamily possess several post-translational lipid modifications at the C terminus (7). Lipidation is believed to have not only targeting but also regulatory function. Unfortunately, isolation of the native and completely lipidated H-Ras (e.g. by baculoviral expression from Sf9 cells) results in very limited yields accompanied by the hydrolysis of the thioester bonds and loss of lipid residues. A combination of chemical and biochemical methods provided access to differently modified Ras proteins that exhibited properties of native Ras proteins (24). We used in this study full-length H-Ras protein purified from E. coli that was additionally farnesylated in vitro. This lipidated Ras was fully active with respect to transformation of PC12 cells following microinjection (24).

Whereas isolated RBD fragments did not discriminate between farnesylated and nonfarnesylated H-Ras, Ras-RAF interaction studies using full-length C-RAF preparations (C-RAF WT and C-RAF-R/L; see also Fig. 4) revealed that C-RAF couples exclusively with farnesylated H-Ras-GTP. The $K_D$ values for full-length C-RAF and activated C-RAF (C-RAF-R/L) were in the range between 0.46 and 0.86 μM (Table 1) and, thus, about 20–40-fold higher than $K_D$ values measured for Ras-RBD interactions. A similar relationship has been reported for interactions between p21-activated protein kinase and small GTP-binding protein Cdc42 in its GTP-bound conformation (45). Although Cdc42 bound to the isolated regulatory fragment with high affinity ($K_D = 18 \text{ nm}$), it associated with full-length p21-activated protein kinase with significantly lower affinity ($K_D = 0.6 \text{ μm}$).

The difference in binding of farnesylated Ras to nonactivated and Ras/Lck-activated C-RAF is surprising if we consider the reported antagonism between Ras binding and 14-3-3 binding to the internal (Ser259) site (46) and the decrease in Ser259 phosphorylation in active C-RAF (47). On the other hand, we have previously shown that chronically stimulated C-RAF from Sf9 cells expressing C-RAF-Y340D/Y341D is multiply phosphorylated at mitogen-activated protein kinase phosphorylation sites between CR2 and CR3 (48), rendering this molecule resistant to Ras interaction (49). These latter data are consistent with our in vitro findings, since we have purified C-RAF from Sf9 cells co-expressing Ras and Lck 48 h postinfection.

For the association of B- and C-RAF with the GDP form of farnesylated Ras, only a low affinity interaction was detected. This observation is in accordance with published data (17, 18), where the authors show that the extreme C terminus of H-Ras and its farnesylned moiety interact with low affinity with isolated CRD independently of GDP or GTP loading state. Our data comprising interactions of the complete B- and C-RAF with farnesylated H-Ras-GDP reveal that these interactions appear to be similar to those reported for isolated CRD ($K_D$ values of $\sim 10 \text{ versus } 20 \text{ μm}$, data not shown).

In this study, we used Ras as a sensor for RAF conformation analysis and found that there are clear differences between B- and C-RAF. First, B-RAF did not discriminate between farnesylated and nonfarnesylated H-Ras, and second, B-RAF coupled to Ras with significantly higher affinities compared with C-RAF (Fig. 4 and Table 1). Interestingly, truncation of the N-terminal tail in B-RAF yielded a protein that possessed in principle the same properties as C-RAF, indicating that the first
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FIGURE 9. Proposed model for the activation-inactivation pathways of B- and C-RAF. Specific association of RAF kinases with membrane lipids results in translocation of cytosolic RAF followed by displacement of 14-3-3 proteins (30). Stimulation of growth factor receptors results in formation of GTP-bound Ras. Transition from the inactive to the active membrane-associated form of RAF requires binding to farnesylated Ras-GTP. Due to the mode of complex formation of B-RAF with 14-3-3 proteins, we propose that B-RAF may exist in the cytosol in an open and a closed conformation. Our model illustrates that B-RAF, contrary to C-RAF, may be also accessible for nonfarnesylated Ras in the cytosolic environment. In accordance with our data, Terai and Matsuda (43) demonstrated recently by means of FRET measurements that B-RAF binds C-terminally truncated H-Ras in the cytosol. We therefore propose that both cytosolic B-RAF conformations possess the ability to associate with Ras-GTP, probably due to the prolonged N terminus.

98 residues of B-RAF may be jointly responsible for its extraordinarily high basal kinase activity as observed in numerous cell lines. Searching for possible explanations for this unusual behavior in Ras binding, it is tempting to speculate that besides the N-terminal fragment of B-RAF, 14-3-3 proteins may play a role in this process as well (6). Moreover, it is possible that the extended N terminus of B-RAF may influence the mode of B-RAF-14-3-3 complex formation. Both RAF kinases, B- and C-RAF, possess two typical 14-3-3 binding sites surrounding C-RAF serines 621 and 259 and serines 729 and 365 in B-RAF. However, although the 14-3-3 protein binding motif surrounding serines 621 and 729 in C- and B-RAF, respectively, is highly conserved (RSpSEP, where pS represents phosphoserine), the sequence surrounding serine 365 in B-RAF (RSSpSAP) differs significantly from the corresponding 14-3-3 binding motif in C-RAF (RSTpSTP). It is possible that the 14-3-3 protein isoforms bind with lower affinity to the position pS365 than to the pS259 binding site in C-RAF. This would suggest that in the case of B-RAF, the complex with 14-3-3 proteins preferentially exists in an open state and thus facilitates cross-linking of B-RAF with C-RAF (and possibly A-RAF). Recently, Terai and Matsuda (43) reported that the removal of the N-terminal part of B-RAF impairs heterodimerization between B- and C-RAF, suggesting a novel mechanism for RAF dimer formation. In contrast to these data, we show here (see Fig. 6B) in accordance with previously published data (5, 41) that the truncation of the first 98 residues of B-RAF did not affect the dimer formation between B- and C-RAF. Furthermore, we demonstrate here that RAF dimerization is Ras-dependent and requires 14-3-3-mediated cross-linking at the C terminus of C-RAF in agreement with previous data (5, 41). Consistent with these observations, Rushworth et al. (42) demonstrated recently that increased amounts of 14-3-3 proteins enhance RAF heterodimerization. Moreover, these authors show that the C terminus of B-RAF, rather than the N-terminal part, regulates the RAF dimer formation, since phosphorylation of threonine 753 at the B-RAF C terminus promoted the disassembly of B/C-RAF heterodimers (42).

An additional 14-3-3 binding site in C-RAF surrounding serine 233 has also been characterized (50). It is questionable, however, whether the corresponding domain in B-RAF serves as a 14-3-3 binding domain, since the basic residue in position −3 is not present in B-RAF. Finally, an atypical 14-3-3 binding site positioned at the C-terminal part of C-RAF-CRD and close to RBD (comprising the residues RKT in positions 143–145) has been described (20). In the vicinity of this
l lipid binding (28, 29) remain constitutively active due to uncontrolled association with unprocessed Ras proteins.

Furthermore, some other members of Ras family proteins distinct from H-, N-, and K-Ras should be considered as potential coupling partners for B-RAF kinase. The GT-Pase Rap1A has been reported to associate with and perhaps activate B-RAF, although there are contradictory reports in the literature (52, 53). Rap proteins are, in contrast to H-, N-, and K-Ras, geranylgeranylated at the C terminus, suggesting that the presence of a farnesyl residue may not be required for B-RAF activation. Besides Rap1 there are several other intriguing candidates, such as members of Ras protein family RIT1/RIT2, NKIRAS1/NKIRAS2, RERG, and RASL11/RASL12, that have been found to lack the typical lipidation motifs and are not subject to any known lipid modifications. The Rit branch of Ras family proteins (RIT1 and RIT2) reveals, additionally, similar effector binding surfaces compared with H-, N-, or K-Ras. Rit proteins have been shown to be required for the outgrowth of neurites in PC12 cells and to stimulate initiation, elongation, and branching of neurites via B-RAF (11). Importantly, dominant-negative B-RAF but not dominant-negative C-RAF (full-length kinase-dead molecules) block Rit-induced neurite elongation and branching but not neurite initiation (54). Thus, besides farnesylated H-Ras that may not exist in sufficient levels in some cells, nonlipidated Rit has been established as a physiological activator of cytosolic B-RAF. These examples demonstrate that there are Ras proteins that associate with B-RAF even without involvement of isoprenyl residues and therefore presumably in the cytosol.

Finally, we found that even in the case of B-RAF, the mode of association with Ras depends on the proximate environment that surrounds the coupling molecules. We show here that in the presence of artificial membranes, only farnesylated Ras had access to both B- and C-RAF. These results are not surprising, since it has been reported that Rap kinases possess several domains that are capable of association with membrane lipids (21, 40). Since the CRD associates with some particular lipids in plasma membranes, it is reasonable to assume that the competition between farnesyl residue and membrane lipids takes place in the course of Ras/RAF coupling at the membranes. Furthermore, it has been shown that in vivo only prenylated Ras proteins are able to associate with membranes (23). It seems likely that for the effective Ras/RAF coupling in the plane of the membranes, a prior contact of the farnesyl residue with the membranes is necessary. This assumption is, however, more valid for C-RAF activation, since B-RAF translocates very poorly to the plasma membrane, as demonstrated by our colocalization studies (see Figs. 8 and S1).

In conclusion, our data support a model of B- and C-RAF activation illustrated in Fig. 9, in which the interaction of farnesyl group with the CRD may represent the primary binding event keeping Ras-GDP and nonactive RAF together, an event that is thought to take place in the cholesterol-rich membrane domains. Following cell stimulation, a high affinity interaction between Ras-GTP and RBD takes place. The resulting Ras-GTP/RAF complex migrates to the nonraft microdomains, as previously suggested (55). We propose furthermore that the major difference between cytosolic B- and C-RAF may exist in the mode of RAF-14-3-3 complex formation, whereby B-RAF exhibits higher affinity for Ras. A more complex model, supported by the recent contribution of Rajalingam et al. (56) includes prohibitin, a protein that has been shown to be necessary for RAF-MEK-ERK activation by Ras. Prohibitin seems to be attached to the C-RAF regulatory region surrounding serines 296 and 301 (48). It is tempting to speculate that the association of prohibitin with serines 296 and 301 in its nonphosphorylated state may increase C-RAF activation via displacement of 14-3-3 proteins from the phosphoserine 259 position (and possibly from CRD), thus allowing better access of Ras to the RBD.

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