Regulation of RAF Activity by 14-3-3 Proteins

RAF KINASES ASSOCIATE FUNCTIONALLY WITH BOTH HOMO- AND HETERODIMERIC FORMS OF 14-3-3 PROTEINS

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Mammalian 14-3-3 proteins play a crucial role in the activation process of RAF kinases. However, little is known about the selectivity of the mammalian 14-3-3 isoforms with respect to RAF association and activation. Using mass spectrometry, we analyzed the composition of the 14-3-3 isoforms attached to RAF kinases and found that B-RAF associates in vivo with 14-3-3 at much higher diversity than A- and C-RAF. We also examined in vitro binding of purified mammalian 14-3-3 proteins to RAF kinases using surface plasmon resonance techniques. While B- and C-RAF exhibited binding to all seven 14-3-3 isoforms, A-RAF bound with considerably lower affinities to ε, τ, and σ 14-3-3. These findings indicate that 14-3-3 proteins associate with RAF isoforms in a pronounced isoform-specific manner. Because 14-3-3 proteins appear in dimeric forms, we addressed the question of whether both homo- and heterodimeric forms of 14-3-3 proteins participate in RAF signaling. For that purpose, the budding yeast Saccharomyces cerevisiae, possessing only two 14-3-3 isoforms (BMH1 and BMH2), served as testing system. By deletion of the single BMH2 gene, we found that both homo- and heterodimeric forms of 14-3-3 can participate in RAF activation. Furthermore, we show that A-, B-, and C-RAF activity is differentially regulated by its C-terminal and internal 14-3-3 binding domain. Finally, prohibitin, a scaffold protein that affects C-RAF activation in a stimulatory manner, proved to interfere with the internal 14-3-3 binding site in C-RAF. Together, our results shed more light on the complex mechanism of RAF activation, particularly with respect to activation steps that are mediated by 14-3-3 proteins and prohibitin.

The serine/threonine-specific RAF kinases play a central role in several normal and pathologic cellular processes including proliferation, differentiation, cell cycle progression, senescence, and apoptosis (1, 2). The first RAF kinase was originally discovered as the oncogenic product of mouse sarcoma virus 3611 (3). Although invertebrates encode only a single RAF kinase, vertebrates express three isoforms, designated as A-, B-, and C-RAF. The C-RAF gene encodes a protein of 648 amino acids that is expressed as a 74-kDa polypeptide (4). A-RAF is a 68-kDa protein showing 60% homology to C-RAF (5). B-RAF is expressed as a full-length protein of 95 kDa or as smaller splice variants (6). All RAF proteins share a similar structure and possess three conserved regions, CR1, CR2, and CR3, that are embedded between variable segments. The CR1 and CR2 domains are part of the regulatory N-terminal half of the RAF proteins, whereas CR3 represents the C-terminal kinase domain. CR1 contains a Ras binding domain and a zinc binding domain, also called cysteine-rich domain. Although all RAF isoforms share a high degree of sequence similarity, they are obviously under different regulation and may have individual functions, mediated by isoform-specific protein-protein interactions (1, 7, 8).

Phosphorylation events are strongly involved in RAF activation process and are subject to tight regulation. Although several phosphorylation sites are well established, RAF phosphorylation remains one of the most controversial aspects of RAF research since the discovery of growth factor-induced tyrosine phosphorylation of C-RAF (9). There are three classes of sites for regulatory phosphorylation: docking sites for 14-3-3 proteins (10), targeting sites (11), and conformation-relevant sites (12). Morrison et al. (13) identified three basal phosphorylation sites in C-RAF; that is, the serine residues at positions 43, 259, and 621. Two of these sites (serine 259 and 621) are involved in binding of 14-3-3 proteins to C-RAF. Phosphorylation of serine 621 seems to be essential for C-RAF activation, as the mutation of serine 621 to alanine resulted in a RAF protein that could no longer be activated by growth factor stimulation (14, 15).

As reviewed by Aitken (20) and by Dougherty and Morrison (21), 14-3-3 proteins accomplish a wide range of functions in the cell. They have been shown to participate in the regulation of such crucial cellular processes as metabolism, signal trans-
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duction, cell cycle control, apoptosis, protein trafficking, transcription, stress responses, and malignant transformation. The regulation of cellular processes by 14-3-3 occurs through several different mechanisms: modulating enzymatic activity, altering protein localization, preventing dephosphorylation, promoting protein stability, inhibiting protein interactions, and mediating protein interactions. Seven mammalian isoforms of 14-3-3 proteins have been identified so far (20, 22, 23). The association of 14-3-3 with client proteins occurs through defined high affinity peptide motifs, two of which (RSXP and RXXXpSXP) are highly conserved and recognized by all 14-3-3 isoforms. In most cases, binding occurs only if a specific serine within the motif is phosphorylated, but some 14-3-3 interactions are independent of phosphorylation (20). A common outcome of 14-3-3 protein binding may be translocation of target proteins into the cytosol. All of the 14-3-3 proteins form homodimers and/or heterodimers that interact with signaling proteins including protein kinase C, RAF kinases, kinase suppressor of Ras (KSR), Cdc25 phosphatases, and BAD pro-
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The abbreviations used are: KSR, kinase suppressor of Ras; PHB, prohibitin; GST, glutathione S-transferase; MS, mass spectrometry; SPR, surface plasmon resonance; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; Bis-Tris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol; MOPS, 4-morpholinepropanesulfonic acid; HPLC, high performance liquid chromatography; ERK, extracellular signal-regulated kinase; shRNA, short hairpin looped RNAs; EF1, elongation factor 1.

All three RAF kinases possess two typical 14-3-3 binding sites surrounding serines 621/259, 729/365, and 582/214 in C-, B-, and A-RAF, respectively. Although the C-terminal 14-3-3 protein binding motif of RAF kinases is highly conserved, the sequence surrounding serine 365 in B-RAF differs from the corresponding 14-3-3 binding motifs in A- and C-RAF (see Fig. 3C). An additional 14-3-3 binding site in C-RAF surrounding serine 233 has also been characterized (26). Furthermore, an atypical 14-3-3 binding site positioned at the C-terminal part of C-RAF-cysteine-rich domain and close to Ras binding domain has been proposed (27). In the vicinity of this 14-3-3 binding site, a contact domain for farnesyl residue of Ras proteins has been identified (28, 29). Therefore, the interaction of farnesyl residue with this domain might be necessary to remove steric hindrances caused by 14-3-3 proteins.

Although direct experimental support is missing, it is generally accepted that the N-terminal regulatory part of RAF interacts in the basal state with the catalytic domain promoting a closed conformation of the kinase. Association with 14-3-3 proteins may further stabilize this inactive conformation (30). Based on more recent information, Rapp et al. (2) suggested a model in which 14-3-3 proteins are necessary for stabilizing the inactive as well as the growth factor-mediated active conformation of RAF. In addition, this model implies that RAF association with plasma membrane lipids (or lipid microdomains called rafts) represents the initial step in the RAF activation process. As a consequence, the association of RAF with membrane lipids and Ras-GTP displaces 14-3-3 from RAF (7, 31). Removal of 14-3-3 proteins from RAF allows access to phosphatases. Regulation of the internal 14-3-3 binding site (Ser(P)-259) by phosphatases has been described by several groups (19, 32, 33). Prohibitin (PHB), a membrane chaperone, influences RAF activation in a positive manner and facilitates 14-3-3 displacement (34). A tentative model has been proposed for the subsequent steps with respect to C-RAF activation that includes Ras-driven B- and C-RAF heterooligomerization (7, 35–37). As reported by our group (35), mutation of the serine 621 to alanine in the C-terminal 14-3-3 binding motif of C-RAF considerably reduced the extent of the heterodimer formation, indicating strongly that 14-3-3 adaptor proteins regulate this process.

In our previous contribution (15) regarding 14-3-3 association with RAF kinases, we were primarily concerned with the characterization of the interactions between 14-3-3ζ homodimer and C-RAF. We examined the kinetics of C-RAF association with 14-3-3 proteins by surface plasmon resonance (SPR) technology and found that the 14-3-3 binding domain surrounding phosphoserine 621 represents the high affinity and probably the major binding site. Time course of endogenous C-RAF activation in mammalian cells upon nerve growth factor stimulation revealed substantial differences between 14-3-3 binding epitopes.

In this study we investigated the putative association of all seven mammalian 14-3-3 isoforms with RAF in vivo and in vitro. By use of mass spectrometry, we analyzed the composition of the endogenously attached 14-3-3 isoforms to A-, B-, and C-RAF proteins and found that B-RAF associates in vivo with a much higher number of 14-3-3 isoforms. Significant differences in binding of 14-3-3 proteins to RAF isoforms have been monitored using the SPR technique. By deletion of the BMH1 gene in S. cerevisiae, we found that both homo- and heterodimeric forms of yeast 14-3-3 proteins participate in RAF activation. Furthermore, we show here that the substitution of serine within the C-terminal 14-3-3 binding site by alanine results in a marked reduction of activity of all three RAF isoforms. In contrast, the mutation of the internal 14-3-3 binding site resulted in elevation of B- and C-RAF activity. The relative binding affinities of the single 14-3-3 binding domains in A-, B-, and C-RAF have been investigated using an indirect competi-
tion assay. Finally, we show here that prohibitin, a scaffold protein affecting C-RAF activation in a stimulatory manner (34), competes for the 14-3-3 binding at the internal 14-3-3 binding site.

**EXPERIMENTAL PROCEDURES**

**Materials**—Benzamidine, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and Nonidet P-40 were obtained from Sigma. Trypsin was from Promega (Mannheim, Germany). Glutathione-Sepharose was purchased from Amersham Biosciences, and nickel-nitrioltriacetic acid-agarose was from Qiagen. Monoclonal anti-phospho-ERK antibodies were from New England Biolabs. Polyclonal anti-A-, B-, and C-RAF antibodies (C20, C19, and C12, respectively), polyclonal anti-green fluorescent protein (FL), and polyclonal anti-14-3-3 antibody (K-19) were obtained from Santa Cruz Biotechnology. Antibody against the pentahistidine tag was obtained from Qiagen.

Phosphospecific antibodies directed against phospho-Ser-259 and phospho-Ser-338 of C-RAF were from Cell Signaling Technology. Phosphospecific antibody directed against phospho-Ser-621 (6B4) has been described previously (15). Epidermal growth factor was obtained from PeproTech GmbH (Hamburg, Germany). Phosphopeptides derived from the 14-3-3 binding domains of A-, B-, and C-RAF containing 14–16 amino acids were purified by high pressure liquid chromatography, and the molecular weight was verified by mass spectroscopy.

**Cloning of Glutathione S-Transferase (GST)-tagged RAF Genes**—For purification of human A-, B-, and C-RAF full-length proteins from mammalian cell lines, a GST tag was introduced. For this purpose RAF proteins have been modified to introduce the recognition sequence for Nhel immediately upstream of ATG codon. In addition, adenine of ATG was converted into cytidine, which changes this codon from methionine into leucine. Modified gene was cleaved with Nhel, and sticky ends were filled in with Klenow enzyme plus dNTPs. After heat inactivation of the polymerase, the RAF-containing fragments were released by further digestion with Xbal and ligated into BamHI-Xbal-cleaved pFastBac-Hta (Invitrogen).

RAF proteins expressed by this system were N-terminal-ex- ligated into BamHI-XbaI-cleaved pFastBac-Hta (Invitrogen). Fragments were released by further digestion with XbaI and treated by 28 amino acids including the GST tag for affinity purification. The recombinant RAF kinases expressed by this system were N-terminal-extend- ed by 28 amino acids including the GST tag for affinity purification on glutathione-Sepharose matrix. This extension did not influence biological properties of RAF kinases in vivo or in vitro. To measure regulation of A-, B-, and C-RAF kinase activities (and/or C-terminal 14-3-3 binding sites (see also Fig. 4) were C-terminal-Myc-tagged and cloned as described in Baljuls et al. (8). The site-specific mutations within the 14-3-3 binding domains were introduced using QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Mutations were confirmed by DNA sequencing.

**Cloning of shRNAs and Production of Lentiviruses**—Short hairpin looped RNAs (shRNAs) directed against human prohibitin-1 (NM_002634) were designed using Invitrogen bioinformatics tools. The following oligonucleotides were employed: hPHB1-shRNA-1-for, 5'-ACC AGC CGT CCC CAA CAC AGC CT TCT CCT TCT GCT CTT CAA GAG AGA GCA GAA GGA AGG CTG TGT TT TTT GGA AAT-3'; hPHB1-
ish peroxidase-conjugated antibodies and detected by enhanced chemiluminescence (ECL, Amersham Biosciences).

**Immunoprecipitation**—HEK293 cells (2 × 10⁶ cells) or Sf9 insect cells (1 × 10⁷ cells) expressing proteins of interest were lysed in 800 μl of Nonidet P-40 lysis buffer supplemented with proteinase inhibitors for 45 min at 4 °C and immunoprecipitated as described previously (8). To assess co-precipitation of 14-3-3 with C-RAF, control and short hairpin PHB cells were serum-starved for 15 h and stimulated with 20 ng/ml epidermal growth factor (Natutec) for 5 min. The cells were lysed in immunoprecipitation buffer (250 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Triton X-100, 1 mM NaVO₃, 10 mM sodium pyrophosphate, 1 mM NaF with protease inhibitor cocktails), and C-RAF was immunoprecipitated using rabbit polyclonal C-12 antibody (Santa Cruz).

**Kinase Activity Measurements**—Kinase assays with RAF samples were performed essentially as described in Hekman et al. (15) using recombinant MEK-1 and ERK-2 as substrates.

**Biosensor Measurements**—To determine quantitatively the interactions between the purified RAF preparations and different 14-3-3 isoforms, the SPR technique was applied. Biosensor Measurements were carried out either on a BIAcore-X or BIAcore-J system (Biacore AB, Uppsala, Sweden) at 25 °C. For that purpose the biosensor chip CM5 was first loaded with anti-GST antibody using covalent derivatization. Purified and GST-tagged 14-3-3 proteins were injected at a flow rate of 10 μl/min, which resulted in a deposition of ~1200 response units. Next the purified His-tagged RAF proteins were injected as indicated. To measure binding of His-tagged 14-3-3 proteins to RAF (see also Fig. 1C) first GST-tagged C-RAF was captured by anti-GST antibody, and subsequently purified 14-3-3 proteins were injected. To measure the competition between RAF and 14-3-3 using synthetic peptides, GST-14-3-3ζ was first captured by anti-GST antibody. Next, RAF proteins were injected in the absence and presence of increasing concentrations of the peptides as indicated in Fig. 3, and the competition between RAF and peptides for 14-3-3 binding was monitored.
Mass Spectrometry Measurements—Samples were separated by SDS-PAGE using NuPAGE Novex 4–12% Bis-Tris gels (MOPS buffer system). Gels were subjected to silver staining (38), and the respective bands were excised and washed according to Shevchenko et al. (39). Briefly, gel pieces were washed 3 times alternately with 50 μl of 50 mM NH₄HCO₃ and 25 mM NH₄HCO₃ in 50% acetonitrile. Subsequently, the gel slices were dried in a vacuum centrifuge. 5 μl of trypsin solution (12.5 ng/μl in 50 mM ammonium bicarbonate) were added to each gel piece and incubated at 37 °C overnight for in-gel digestion. The obtained peptides were eluted with 20 μl of 5% formic acid and subjected to nano-liquid chromatography-MS/MS analysis. Thereby, an Ultimate 3000 nano-HPLC system (Dionex GmbH, Idstein, Germany) was used. The samples were preconcentrated on a 100-μm inner diameter, 2-cm C18 column (nanoseparations, Nieuwkoop, The Netherlands) using 0.1% trifluoroacetic acid with a flow rate of 8 μl/min. The peptides were then separated on a 75-μm inner diameter, 15 cm, C18-PepMap column (flow rate 300 μl/min; Dionex GmbH, Idstein, Germany) using a 1-h binary gradient from 5 to 50% solvent B (solvent A: 0.1% formic acid; solvent B: 0.1% formic acid, 84% acetonitrile). The nano-HPLC was directly coupled to an ion trap mass spectrometer (LCQ DecaXP®, ThermoElectron GmbH, Dreieich, Germany) acquiring repeatedly one full-MS and three tandem-MS spectra of the most intensive ions in the respective full MS scan. The tandem-MS spectra were searched against the NCBI nr database using the Mascot Daemon and the Mascot algorithm (Version 2.1; Matrix Science Ltd., London, UK) using the following adjustments: taxonomy (*Homo sapiens*), trypsin as protease, one missed cleavage site, oxidation of methionine (pyroglutamic acid for N-terminal Gln as variable modifications), 1.5-Da tolerance for MS and MS/MS.

RESULTS

Mammalian 14-3-3 Adaptor Proteins Associate with RAF Kinases in an Isoform-specific and Differential Manner—Although numerous signal transduction processes are regulated by multiple 14-3-3 isotypes (20, 21, 40, 41), the 14-3-3 isotype specificity has not been well defined so far, and no data are available on particular 14-3-3 isoforms involved in RAF signaling. To examine whether mammalian 14-3-3 proteins specifically associate with RAF kinases, we performed mass spectrometry analysis of RAF signaling complexes purified from HEK293 cells and identified several 14-3-3 isoforms closely attached to RAF. As demonstrated in Fig. 1A, both B- and C-RAF interact with 14-3-3ζ and ζ. In addition, B-RAF has been found to be associated with four other 14-3-3 isoforms (β, γ, τ, and η), indicating that B-RAF may possess more versatile functions in cell signaling compared with C-RAF. Similar to C-RAF, A-RAF was accompanied only by two 14-3-3 isoforms (τ and ζ). Furthermore, we identified several heat shock proteins (HSP90, HSP70, Cdc37, and HSP40) closely attached to RAF isoforms (see Fig. 1A). The elongation factor 1 (EF1) has also been detected in these samples. However, as EF1-β also precipitated in the GST control, we cannot definitively assign EF1 to a RAF signaling complex. In contrast to B- and C-RAF, A-RAF associated with tubulin. The results shown in Fig. 1A regarding 14-3-3 association with RAF did not provide further information about the apparent affinity and dimerization modus of the attached 14-3-3 proteins, i.e. binding of both homo- and heterodimeric forms are possible. It should be noted that single recombinant 14-3-3 isoforms, irrespective of their *in vivo* preference, appear as a dimer, as a monomeric form is unstable due to unfavorable thermodynamic properties (23). However, the exact composition of 14-3-3 dimers during the course of RAF activation still remains elusive.

On the other hand, the use of purified recombinant 14-3-3 proteins and RAF kinases allows the assessment of specificity and affinity of the single mammalian 14-3-3 proteins toward RAF. Therefore, we extended our investigations described previously (15) and measured binding of A-, B-, and C-RAF kinases to all seven mammalian 14-3-3 isoforms (β, γ, ε, ζ, τ, ζ, and η). For that purpose GST-tagged 14-3-3 isoforms purified from *E. coli* were immobilized on the biosensor chip. Next, the purified His-tagged RAF proteins were injected. Association-dissociation curves obtained for the interactions between the recombinant 14-3-3 proteins and RAF kinases document significant differences in binding of 14-3-3 isoforms to A-, B-, and C-RAF (Fig. 1B and supplemental Fig. S1). Although B- and C-RAF exhibited binding to all of the seven 14-3-3 isoforms, A-RAF revealed *in vitro* very poor binding to ε, τ, and ζ. 14-3-3. The association rates of 14-3-3 binding to B- and C-RAF are comparable; in contrast, the *k*ₐ of A-RAF binding was lower. The common feature for all of the RAF isoforms was the finding that 14-3-3y bound with highest affinity and 14-3-3ε with lowest affinity among the seven 14-3-3 isoforms. In addition we observed that 14-3-3σ revealed a much higher affinity for
B-RAF compared with A- and C-RAF (Fig. 1B and supplemental Fig. S1). Thus, we show here that mammalian 14-3-3 proteins associate with RAF in a pronounced isofrom-specific manner. The finding that B-RAF, in contrast to C-RAF, associates with numerous 14-3-3 isofroms (Fig. 1A) suggests that B-RAF fulfills multiple functions in the cell, most of them not elucidated yet.

The binding measurements presented in Fig. 1B and supplemental Fig. S1 have been carried out with 14-3-3 homodimers. To examine binding properties of the heterodimeric forms of 14-3-3 proteins regarding association with RAF we isolated a His-tagged 14-3-3 heterodimer consisting of 14-3-3 from Sf9 insect cells, a His-tagged 14-3-3 heterodimer consisting of 14-3-3 binding sites (substitution of serine 365 and 729 by alanine in B-RAF and serine 259 and 621 in C-RAF) and RAF mutants impaired in both 14-3-3 coupling domains (A and B). Expression levels of the RAF proteins and their phosphorylation status were determined by immunoblotting using RAF antibodies and corresponding RAF phosphospecific antibodies as indicated. Kinase activities were measured by a coupled kinase assay (KA) in the presence of recombinant MEK and ERK proteins as described under “Experimental Procedures.” The kinase dead variant of B-RAF (B-RAF-K483M) served as negative control and did not reveal any catalytic activity in yeast. These experiments were repeated three times.

Therefore, to test whether RAF kinases associate in vivo functionally with homo- and heterodimeric forms of 14-3-3 proteins, we used the budding yeast S. cerevisiae. These cells contain only two 14-3-3 homologues, BMH1 and BMH2, thus providing a simple model system for functional studies of 14-3-3 interaction. We also took advantage of a particular strain of S. cerevisiae omitting the BMH2 isoform. BMH2 reveals a high degree of homology with mammalian 14-3-3e.

Upon transformation of wild-type and BMH2-deficient yeast strain with B- and C-RAF, we investigated the association of yeast 14-3-3 proteins with RAF and the consequences for its kinase activity. In this experiment we also used RAF variants that were mutated at the single 14-3-3 binding sites and RAF mutants impaired in both 14-3-3 coupling domains (BMH2, S259A/S621A and B-RAF-S365A/S729A). Importantly, data presented in Fig. 2 demonstrate that C-RAF associates in vitro effectively with both hom- and heterodimeric forms of 14-3-3 proteins. Importantly, also the yeast 14-3-3 homolog BMH1 serves as an acceptable coupling partner for C-RAF (see Fig. 1C).

Putative 14-3-3 Binding Sites of RAF Kinases Differ in Their Binding Affinities—Results presented in Fig. 1 and supplemental Fig. S1 document that mammalian 14-3-3 isofroms bind differentially to RAF isofroms. However, these data provide no information about the binding strength of 14-3-3 to the indi-
vidual binding domains. To assess the binding affinities of 14-3-3 for the C-terminal and internal binding domains in RAF, we developed an indirect competition assay in which we used purified full-length protein components (RAF and 14-3-3 proteins) and synthetic phosphopeptides serving as competitive inhibitors for the RAF/14-3-3 interaction. The phosphopeptides were derived from the corresponding C-terminal and internal 14-3-3 binding domains of A-, B-, and C-RAF. The 14-3-3/RAF association was monitored by SPR technique as described above (see Fig. 1B). Because 14-3-3ζ isoform has been found to interact with similar affinities with all three RAF isoforms, we used this isoform for competition studies. For that purpose, GST-14-3-3ζ protein was first captured by immobilized anti-GST antibody, and RAF association was monitored in the absence of synthetic peptides. This value has been defined as 100% binding. Next, phosphopeptides derived from 14-3-3 binding domains over a range of concentration between 10⁻⁴ and 10⁻⁹ M were mixed with RAF proteins, and the degree of RAF/14-3-3 inhibition was detected. As demonstrated in Fig. 3, peptides corresponding to the C-terminal 14-3-3 binding sites of A-, B-, and C-RAF revealed considerably higher inhibitory potential than peptides derived from the internal binding sites competing with an IC₅₀ value of ~50–100 nM. Thus, data presented in Fig. 3 suggest that the C-terminal 14-3-3 binding domain in RAF represents the high affinity binding site and probably the major binding epitope. These data are in accordance with previously published findings (15). Among C-terminal peptides, no significant differences were monitored. In contrast, peptides corresponding to the internal binding sites exhibited differential inhibitory potential. As illustrated in Fig. 3B, the peptide corresponding to the B-RAF internal binding site revealed the highest affinity for the 14-3-3ζ isoform followed by C- and A-RAF peptides. These results suggest that B-RAF may bind 14-3-3 with the highest binding capacity. These observations are in accordance with mass spectrometry data (Fig. 1A).

**Regulation of RAF Kinase Activity by Internal and C-terminal 14-3-3 Binding Sites Differs for A-, B-, and C-RAF—With respect to RAF activation cycle, 14-3-3 proteins have been found to support RAF activation (14, 42). On the other hand, it has also been reported that 14-3-3 are not essential for RAF function (43). Although the C-terminal 14-3-3 protein binding motif (RSpSEP) of RAF kinases is highly conserved, the sequence surrounding serine 365 in B-RAF (RSpSAP) differs from the corresponding 14-3-3 binding motifs in A- and C-RAF (RSTpSTP, see also Fig. 3C).

To address how phosphorylation of 14-3-3 binding sites influence and regulate the activation cycle of A-, B-, and C-RAF, we replaced the regulatory serines within the 14-3-3 binding domains in A-, B-, and C-RAF by alanine and investigated both activated and non-activated RAF proteins. In accordance with previous data (15) the degree of serine 621 phosphorylation in C-RAF-S259A mutant was reduced, indicating interdependence between these two residues (Fig. 4A). This effect was more pronounced in the samples expressed in Sf9 insect cells (supplemental Fig. S2). On the other hand, the phosphorylation degree of the serine 259 was not considerably influenced by the introduction of alanine in position 621.

**FIGURE 3. Inhibition of RAF binding to 14-3-3ζ by phosphopeptides derived from C-terminal and internal 14-3-3 binding domains of A-, B-, and C-RAF.** Purified and GST-tagged 14-3-3ζ were captured by immobilized anti-GST antibody. Approximately 900 resonance units of 14-3-3 were bound for each measurement. In the next step purified C-RAF (10 pmol) was injected in the presence and absence of phosphopeptides corresponding either to the C-terminal 14-3-3 binding domains (A) or internal 14-3-3 binding domains (B) of A-, B-, and C-RAF. Phosphopeptides were mixed with RAF samples over a range of concentration between 10⁻⁴ and 10⁻⁹ M and injected without incubations. The core sequences of the internal and C-terminal 14-3-3 binding domains of A-, B-, and C-RAF are depicted in C.

Regarding kinase activities of these C-RAF mutants, the substitution of serine by alanine in position 621 (C-RAF-S621A) abolished almost completely the activity (Fig. 4A). In contrast, the C-RAF-S259A mutant revealed highly elevated activity consistent with data previously published (13, 17, 19, 44). Surprisingly, as observed in Sf9 cells (but not in HEK293 cells), in the case of maximal RAF stimulation that can be achieved by co-expression with Ras12V and Lck, the substitution of serine 621 by alanine did not completely prevent the C-RAF activation (supplemental Fig. S2). Similar effects were observed with the doubly mutated C-RAF (C-RAF-S259A/S621A). The increased phosphorylation at position S338 (and tyrosine phosphorylation as well) partially compensates for impaired 14-3-3 binding in these RAF mutants expressed in the insect cells. These findings together indicate that 14-3-3 association is critical for regulation of RAF activation process.
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A

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B

C

FIGURE 4. Analysis of the phosphorylation state of 14-3-3 binding sites in A-, B-, and C-RAF and changes of kinase activities caused by mutations within the 14-3-3 binding domains. To determine the extents of phosphorylation at the 14-3-3 binding sites, the phosphospecific antibodies directed against phosphoserines 259 and 621 (6B4 antibody) of C-RAF have been used for all three RAF isoforms as these antibodies cross-link with adequate positions in A- and B-RAF. In A and B the crude lysates from HEK293 cells transfected with C- and B-RAF and the indicated mutant proteins were subjected to SDS-PAGE and blotted on nitrocellulose. Expression levels of RAF proteins and their phosphorylation status were detected with different antibodies as indicated in the figure. Kinase activities were measured directly using crude lysates in the presence of recombinant MEK and ERK proteins. ERK phosphorylation was detected by phosphospecific anti-ERK antibody. IP, immunoprecipitate; IB, immunoblot. C, due to its low basal activity, A-RAF wild type (Myc-tagged) and the indicated A-RAF mutants impaired in 14-3-3 binding were immunoprecipitated from HEK293 cell lysates by an anti-Myc antibody. The kinase activity measurements were performed using immunoprecipitated material attached to protein-G-agarose as described under "Experimental Procedures." Stimulation of A- and C-RAF have been performed either with epidermal growth factor (EGF, 100 ng/ml) for 5 min or by co-expression with Ras12V and Lck. These experiments were carried out three times.

B-RAF behaves in general differently from A- and C-RAF. It exhibits very high basal activity even in the absence of cell stimulation. In contrast to C-RAF, we did not register an interdependence between these two 14-3-3 binding domains, as the degree of serine 365 and 729 phosphorylation was not significantly altered upon introduction of alanine in positions 365 or 729. In accordance with data recently published by Brummer et al. (45), we observed a complete abolishment of kinase activity in B-RAF-S729A mutant and a strong increase in activity caused by introduction of alanine within the internal 14-3-3 binding site (Fig. 4B). The presence of Ras12V and Lck did not elevate the kinase activity of the B-RAF-S729A mutant, as no further tyrosine phosphorylation could be achieved by Src kinases (data not shown).

Importantly, the oncogenic form of B-RAF (B-RAF-V600E variant) behaves contrary to B-RAF wild type. The replacement of serine 729 by alanine caused only partial reduction of the kinase activity, indicating that B-RAF-V600E mutant does not necessarily require an intact C-terminal 14-3-3 binding site for maintenance of its catalytic activity. With respect to regulation of kinase activity by internal 14-3-3 binding site, no dramatic differences were observed between B-RAF-600E and B-RAF-V600E/S365A mutant; substitution of serine 365 by alanine enhanced the kinase activity of B-RAF-V600E only moderately. Results obtained with B-RAF-V600E are reminiscent of effects observed with B-RAF wild type expressed in yeast (Fig. 2A), where the substitution of serine 729 by alanine did not alter its kinase activity.

Although A-RAF contains two typical 14-3-3 binding domains that are similar to the 14-3-3 domains in C-RAF, little is known about the binding and regulation of this kinase by 14-3-3 isoforms. We show here that A-RAF wild type is phosphorylated at these positions in both activated and non-activated samples (Fig. 4C). Similar to C-RAF, the replacement of serine 214 in A-RAF by alanine led to a dramatic reduction of serine 582 phosphorylation (Fig. 4C), indicating a strong interdependence between these two 14-3-3 binding sites.

The presence of the serines 621 and 729 in C- and B-RAF, respectively, has been shown to be important for the effective activation of these kinases. We asked whether the phosphorylation of the corresponding serine in A-RAF (Ser-582) plays a similar role. The results shown in Fig. 4C document that A-RAF behaves differentially compared with B- and C-RAF. The presence of the intact C-terminal 14-3-3 binding domain was only partially required for effective stimulation (Fig. 4C). Investigating the role of the internal 14-3-3 binding site in A-RAF, we found that the kinase activity of the A-RAF-S214A variant was highly reduced even in the presence of Ras and Lck (Fig. 4C). These results indicate that similar to B- and C-RAF, the activation of A-RAF is strongly influenced by the internal 14-3-3 binding domain; however, in the opposite direction. A possible explanation for this effect may be the finding that A-RAF-S214A mutant revealed almost no phosphorylation at the position serine 582 (see Fig. 4C). In summary, we show here that the functional roles of the internal and C-terminal 14-3-3 binding domains, although both representing classical 14-3-3 binding sites, differ substantially from each other.

Prohibitin Displaces 14-3-3 in C-RAF from the Internal 14-3-3 Binding Site Leading to Elevation of Kinase Activity—We recently reported that PHB, a ubiquitously expressed membrane-associated protein (46), influences in vivo RAF
activity in a positive manner. Specifically, we showed that the reduction of PHB content in the cell causes increased association of 14-3-3 to C-RAF, suggesting that PHB might be required for the displacement of 14-3-3 in the course of RAF activation (34). Similar results were obtained by infection of HeLa cells with lentiviruses carrying short hairpin RNA directed against prohibitin-1 (see supplemental Fig. S3). However, whether PHB influences 14-3-3 displacement directly or indirectly by membrane tethering remained unclear. To test the possibility whether PHB acts as a direct inhibitor for C-RAF association with 14-3-3 proteins, we performed SPR analysis. For that purpose, we immobilized first the purified and GST-tagged 14-3-3 to the CM5 chip as described under "Experimental Procedures." Next, we measured binding of C-RAF to 14-3-3. To examine whether prohibitin may affect the association of C-RAF with 14-3-3 proteins, we preincubated C-RAF with increasing amounts of prohibitin and monitored the possible changes in binding to 14-3-3. As depicted in Fig. 5, the addition of PHB considerably impaired the C-RAF binding to 14-3-3, indicating that PHB and 14-3-3 compete for C-RAF binding. Importantly, in contrast to C-RAF-S621A mutant, the association of the C-RAF-S259A mutant with 14-3-3 was affected only to low degree by the addition of PHB (Fig. 5A). Surprisingly, testing all seven mammalian 14-3-3 proteins, we registered pronounced isoform specificity (see Fig. 5B). In contrast to 14-3-3β, -γ, and -ζ, 14-3-3 γ could not be displaced by PHB. On the other hand, binding of 14-3-3ε and 14-3-3σ to C-RAF was completely inhibited by PHB. The degree of this inhibition was comparable with the control value that could be achieved by the addition of a high concentration (20 μM) of the Ser(P)-621 peptide (see also Fig. 3A). To prove the in vivo interaction between C-RAF and PHB, we used HEK293 cells expressing green fluorescent protein-tagged C-RAF wild type and 14-3-3 binding mutants together with His-tagged PHB. In agreement with the in vitro data presented in Fig. 5A, the amount of PHB co-precipitating with C-RAF was considerably increased in the case of the S259A mutant that is impaired in 14-3-3 binding at the internal site (Fig. 5C). Together these results support the view that PHB-mediated activation of C-RAF at membranes occurs by direct 14-3-3 displacement most probably from the internal 14-3-3 binding site.

**DISCUSSION**

Although the participation of 14-3-3 proteins in RAF signaling has already been described in 1995 by Luo et al. (47), to our knowledge no data are available on specificity and affinity of particular mammalian 14-3-3 isoforms in RAF signaling process so far. Our previous studies concerning association of 14-3-3 proteins with the internal and C-terminal 14-3-3 binding sites of C-RAF (15) were restricted to the 14-3-3ζ isoform and C-RAF kinase.

To address the question of whether mammalian 14-3-3 isoforms reveal specificity in association with RAF kinases, we performed here systematic binding studies using purified preparations of all seven mammalian 14-3-3 isoforms and RAF isozymes (A-, B-, and C-RAF). Data obtained by both direct binding (Fig. 1B and supplemental Fig. S1) and by competition assays (Fig. 3) document unambiguously that 14-3-3 proteins interact with RAF and vice versa in an isoform-specific manner. As the commercially available antibodies directed against mammalian 14-3-3 isoforms reveal cross-reactivity between individual isoforms, we took advantage of mass spectrometry to identify RAF-associated 14-3-3 proteins. Surprisingly, B-RAF associated with a much higher number of 14-3-3 isoforms compared with A- and C-RAF (see Fig. 1A). The 14-3-3ε isoform was present in each sample, indicating that 14-3-3 proteins partially appear in heterodimeric form. Data showing that A-, B-, and C-RAF display in vivo differential composition of associ-
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14-3-3 proteins go along with findings that RAF isoforms fulfill different functions in the cell due to their subcellular localization. Although C-RAF has been detected predominantly at the plasma membrane, B-RAF localizes mainly in the cytosolic environment (7), explaining the high amount of attached 14-3-3 proteins. The fact that A-RAF associates with tubulin and the finding that A-RAF localizes predominantly at the endosomal vesicles are consistent with a unique function of this RAF isoform within the RAF family of kinases.4

Remarkably, using MS techniques, the scaffold protein KSR, which interacts with the core kinase components of the ERK cascade, has been found to be associated also with a higher number of mammalian 14-3-3 isoforms (33). Besides 14-3-3ε, three other isoforms (14-3-3γ, -ζ, and -β) were detected in a KSR sample immunoprecipitated from COS7 cells. Interestingly, in vitro binding assays performed by our group with purified KSR and 14-3-3 proteins identified these three isoforms as high affinity binding partners (data not shown). Although KSR possesses a conserved kinase domain, no catalytic activities could be detected. On the other hand, KSR has been shown to associate directly with C-RAF in a growth factor-inducible manner (48). Therefore, it is reasonable to assume that the relatively high number of KSR-associated 14-3-3 proteins may serve to cross-link KSR with its client partners such as C-RAF.

In analogy to KSR, diverse 14-3-3 isoforms attached to B-RAF may have different functions. It is obvious from data presented in Fig. 4 that 14-3-3 proteins are primarily crucial for B-RAF stimulation, because the substitution of serine 729 by alanine within the C-terminal 14-3-3 binding site inhibited completely the activation of B-RAF. However, as B-RAF has been reported to form heterodimeric complexes with C-RAF (and possibly with A-RAF as well) in a Ras- and 14-3-3-dependent manner (35–37), we propose that at least some of the associated 14-3-3 isoforms are involved in the cross-linking of B-RAF with other signaling molecules. In this context a complex formation between B-RAF and KSR could also be considered. However, no experimental data on B-RAF interaction with KSR are available so far. Contrary to B-RAF, C-RAF purified from HEK293 cells (see Fig. 1A) has been found to be associated only with 14-3-3ε and -ζ. Obviously, the presence of these two isoforms is sufficient for activation of C-RAF isoform. This assumption is in accordance with the fact that several invertebrates such as Drosophila melanogaster and Caenorhabditis elegans possess only two 14-3-3 isoforms that are sufficient for regulation of the Ras/RAF/MAPK cascade. Moreover, we addressed the issue of whether an individual 14-3-3 homodimer possesses the ability to activate RAF in vivo. To this end, the budding yeast S. cerevisiae, possessing only two 14-3-3 isoforms, served as the testing system. By deletion of the BMH2 gene we were able to show that the activation of both B- and C-RAF could be achieved in vivo by a single 14-3-3 isoform (see also Fig. 2). Based on these results, we suggest that whereas a single homodimeric form of 14-3-3 is sufficient for RAF activation, the function of heterodimeric forms of 14-3-3 may be rather to cross-link other RAF isoforms or KSR. In addition, the formation of sandwich structures between RAF and 14-3-3 that has been proposed for silencing RAF activity would also require heterodimeric forms of 14-3-3 proteins due to the different structures and binding affinities of the internal versus C-terminal 14-3-3 binding domains.

Regarding regulation of RAF activity by 14-3-3 proteins, we show here that the intact C-terminal 14-3-3 binding site is in general necessary for effective RAF activation. On the other hand, regulation of the RAF activity by the internal 14-3-3 binding site differs among the RAF isoforms. Although the substitution of serine 259 and 365 by alanine in C-RAF and B-RAF caused a dramatic enhancement of the basal kinase activity (Fig. 4, A and B), the analogous A-RAF mutant (A-RAF-S214A) revealed a reduction of activity compared with A-RAF wild type (Fig. 4C). This observation could be explained by the extremely low degree of serine 582 phosphorylation (Fig. 4C) in A-RAF-S214A mutant, thus preventing interactions with 14-3-3 proteins. Other opposite behaviors of A-RAF kinase regarding its activation should be taken into account as well, e.g. differential regulation of kinase activity by N region (8) and isoform-specific hinge segment domain (49).

In this context recently published findings by two independent groups concerning developmental disorders caused by mutations within the 14-3-3 binding domains in C-RAF are of particular interest (50, 51). Most of the altered residues in B- and C-RAF that are associated with cancer have been found localized within the kinase domain (for review, see Wellbrock et al. (1)). Pandit et al. (50) and Razzaque et al. (51) reported for the first time on pathological C-RAF mutations positioned within the internal 14-3-3 binding site and in the proximity of the C-terminal 14-3-3 binding domain. These mutations led to activation of C-RAF. The consequences of this gain-of-function are severe cardio-facio-cutaneous disorders called Noonan and LEOPARD syndrome. Most of these mutations altered the motif flanking serine 259 of the internal 14-3-3 binding domain. Displacement of the serine 259 by phenylalanine (S259F) abolishes the autoinhibitory mechanism of C-RAF, resulting in a permanent active C-RAF form. Taken together, our results and data presented by Pandit et al. (50) and Razzaque et al. (51) corroborate the importance of 14-3-3 binding sites in C-RAF for correct regulation of signal transduction in the Ras/RAF/MAPK cascade.

We explore here also the functional consequences of the mutations of crucial serines within the 14-3-3 binding domains of the oncogenic B-RAF-V600E variant (see also Fig. 4B). Substitution of the valine 600 by glutamic acid within the activation segment of B-RAF leads to a highly activated B-RAF kinase that could not be further activated (1, 12). Notably, in about 70% of malignant melanomas, the hyperactive B-RAF-V600E was detected (52, 53). On the other hand, it has been recently shown that C-RAF activity was not altered by the analogous V492E mutation and is not transforming (54). A similar observation has been made with A-RAF-V453E mutant. Surprisingly, as shown in Fig. 4B, even in the case of doubly mutated B-RAF (B-RAF-S365A/S729A) the activity was only slightly reduced by serine to alanine exchange in these positions. These data corroborate the robust nature of oncogenic B-RAF and demonstrate no need of 14-3-3 proteins for activation and mainte-

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nance of activity of this mutant. Interestingly, similar effects have been observed with B-RAF wild type expressed in yeast. As presented in Fig. 2A, the substitution of serine 729 by alanine did not lead to B-RAF inactivation. This effect could be explained by a putative phosphorylation of the activation loop at the position threonine 599 by an yet unknown kinase in yeast, thus mimicking the active conformation as observed in the B-RAF-V600E variant (12).

An additional aspect concerning the promotion of C-RAF activity by 14-3-3 should be taken into account as well. Recently, we showed that PHB is a positive regulator of C-RAF activity. Reduction of PHB content in the cell causes increased phosphorylation of serine 259 and results in elevated 14-3-3 activity. Reduction of PHB content in the cell causes increased 14-3-3 binding directly or indirectly by membrane tethering remained to be investigated. To this end, we used here an in vitro competition assay with purified components and demonstrate that PHB interferes indeed with the binding of 14-3-3 to C-RAF (see Fig. 5). The inhibitory effects were more pronounced by testing the C-RAF-S621A mutant, indicating that PHB contributes to RAF activation by removal of 14-3-3 from the internal 14-3-3 binding site. Importantly, in agreement with in vitro data, experiments performed in vivo revealed that PHB associates tightly with a C-RAF mutant impaired in 14-3-3 binding (Fig. 5C).

These data support our working hypothesis illustrated in Fig. 6 proposing that the removal of 14-3-3 from the internal binding site in C-RAF is facilitated by PHB at the plasma membrane. In this scenario, however, PHB does note affect the inactive C-RAF that is supposed to exist as a stable complex with the dimeric form of 14-3-3 proteins in the cytosolic environment. In the next activation step (not shown in Fig. 6) 14-3-3 dimers may dissociate completely from RAF. However, temporary cross-linking with KSR and/or B-RAF should also be considered.

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