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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Andreas Fischer1, Angela Baljuls2, Joerg Reinders3, Elena Nekhoroshkova4, Claudia Sibilski4, Renate Metz5, Stefan Albert5, Krishnaraj Rajalingam6, Mirko Hekman6, and Ulf R. Rapp1,2

From the 1Bayerisches Krebsforschungszentrum, University of Wuerzburg, 97078 Wuerzburg, Germany, the 2Institute of Functional Genomics, University of Regensburg, 93053 Regensburg, Germany, and the 3Emmy Noether Group of the Deutsche Forschungsgemeinschaft, Institute for Biochemistry II, Goethe University Medical School, 60596 Frankfurt, Germany

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 €, r, and $ 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). In contrast, exchange of serine 259 by alanine or aspartic acid resulted in enhancement of kinase activity, indicating that phosphorylation of serine 259 is inhibitory (13, 16–19). Furthermore, the binding of 14-3-3 proteins to the C-terminal conserved site has been found to be differentially and dynamically regulated (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-
Regulation of RAF Activity by 14-3-3 Proteins

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*SXP 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),

3 Cdc25 phosphatases, and BAD pro

naling proteins including protein kinase C, RAF kinases, kinase suppressor of Ras (KSR), 3 Cdc25 phosphatases, and BAD protein (24). The pattern of the dimer formation for two of the most abundant mammalian isoforms, ε and γ 14-3-3, has been investigated by Chaudhri et al. (23). 14-3-3-γ has been found to be present in homodimeric form, but also heterodimers could be formed mainly with 14-3-3ε. In contrast, 14-3-3ε formed heterodimers with 14-3-3β, -γ, -ζ and -η, but no homodimers were detected (23). The existence of the 14-3-3 homodimers and particular combination of the heterodimers in vivo may have important implications for function of 14-3-3 proteins serving either as a chaperone or adaptor protein. However, little is known about the specificity of the putative dimer combinations. Importantly, the budding yeast Saccharomyces cerevisiae contains only two 14-3-3 homologues, BMH1 and BMH2, as compared with mammalian and plant cells that express 7–14 isoforms, thus, providing a simple model system for functional studies of 14-3-3 interactions with other signaling molecules. Recently, by use of mass spectrometry, a detailed proteomic analysis of in vivo 14-3-3 interactions of the yeast 14-3-3 proteins has been performed yielding at least 271 association partners (25). These findings reflect the important and the complex role of the 14-3-3 proteins in the cell.

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 competitiv

3 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-(hydroxyethyl)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.
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-nitriotriacetic 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, phospho-Thr-328, 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 NheI 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 NheI, 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-extended 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 by 14-3-3 proteins, RAF wild type and RAF mutants possessing serine to alanine substitutions within the internal 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 QuickChange 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 ACG CTG CCC CAA CAC AGC CCT CTC TCT GCT CTT CAA GAG AGA GCA GAA GGA AGG CTG TGT TTT TTT GGA AA-3′; hPHB1-shRNA-1-rev, 5′-TAT CGA TTT CCA AAA AAA CAC AGC CTT CCT TCT GCT TCT TGA AGA GCA GAA GGA AGG CTG TGT TGG GGA-3′.

The oligos were cloned to PLVTHM vector (kindly provided by Prof. Didier Trono, EPFL; Switzerland). Production of lentiviruses and infection of HeLa cells were performed as described on the tronolab website. The green fluorescent protein-expressing (shRNA-carrying) HeLa cells were sorted out by fluorescence-activated cell sorter, and the efficiency of the knockdown was monitored by immunoblot and reverse transcription-PCR analysis. Several clones were tested, and the clones with the best knockdown were employed for further studies. The control cells were infected with lentiviruses carrying empty vector (PLVTHM) alone. These cells were cultured in RPMI medium in the presence of 10% fetal calf serum and antibiotics.

Purification of RAF Kinases, Prohibitin, and 14-3-3 Proteins, SDS-PAGE, and Western Blot Analysis—For the production of recombinant RAF kinases, the Sf9 cells were infected with the desired baculoviruses at a multiplicity of infection of 5 and incubated for 48 h at 30 °C. The cells were then washed with phosphate-buffered saline and pelleted at 250 × g for 15 minutes. Lysis and purification of the GST- and His-tagged RAF kinases from Sf9 cells was performed as described in Hekman et al. (15). For the purification of the GST-tagged RAF kinases from HEK293 cells, the same procedure as described above was applied. To remove 14-3-3 proteins associated with RAF, some purification procedures were carried out in the presence of 1.0% Empigen BB (Calbiochem). All seven mammalian 14-3-3 isoforms were expressed in Escherichia coli as GST fusion proteins using pGEX-2T vector (GE Healthcare) and purified by glutathione-Sepharose affinity chromatography. To purify the heterodimeric forms of 14-3-3 proteins, Sf9 insect cells were used as the expression system. For that purpose the Sf9 cells were infected e.g. with baculoviruses containing both His-tagged 14-3-3ε and non-tagged 14-3-3ζ. The desired 14-3-3 heterodimer was isolated by nickel-agarose affinity purification as described above. The homodimeric forms of 14-3-3ε and 14-3-3ζ (both His-tagged) were purified from Sf9 cells as control samples in the same way as performed with heterodimers. To prove whether 14-3-3 proteins purified from Sf9 insect cells (and E. coli) exist in dimeric form, we performed gel filtration chromatography using an AKTA-System (GE Healthcare). For that purpose, 14-3-3 samples obtained by nickel-agarose chromatography were applied to a Superdex 200 column (GE Healthcare) and eluted by 25 mM Tris-HCl, pH 7.4, and 150 mM NaCl. All of the investigated 14-3-3 protein samples migrated at ~70 kDa, indicating dimer formation. In these samples no monomeric forms of 14-3-3 were detected. Prohibitin was purified from E. coli essentially as described previously (34). The purity of RAF kinases, prohibitin, and 14-3-3 proteins was documented by SDS-PAGE (10% gels) and staining with Coomassie Blue. For Western blot analysis the gels were transferred to nitrocellulose membranes (Schleicher & Schuell) and probed either with phosphospecific antibodies or with antibodies specific for A-, B-, and C-RAF and phospho-ERK as indicated in the legends to the figures. After the washing procedure, the membranes were incubated with specific secondary horserad-
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.
Regulation of RAF Activity by 14-3-3 Proteins

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 respectively 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 DecaXPPlus, 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 NCBlnr data base using the Mascot Daemon and the Mascot algorithm (Version 2.1; Matrix Science Ltd., London, UK) using the following adjustments: taxonomy (Hom 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 analysis 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, τ, ε, ζ, η, and ζ). For that purpose GST-tagged 14-3-3 isoforms purified from E. coli were immobilized on the biosensor chip. Next, the purified and His-tagged RAF proteins were injected. Association-dissociation curves were 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_on for A-RAF binding was lower. The common feature for all of the RAF isoforms was the finding that 14-3-3-y bound with highest affinity and 14-3-3ε with lowest affinity among the seven 14-3-3 isoforms. In addition we observed that 14-3-3ζ revealed a much higher affinity for

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 isoforms (20, 21, 40, 41), the 14-3-3 isotope 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 14-3-3ε 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 ε). 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.

FIGURE 1. Differential association of mammalian 14-3-3 isoforms with RAF kinases. A, mass spectrometry analysis of RAF-associated proteins in vivo. The GST-tagged RAF kinases isolated from HEK293 cells by glutathione-Sepharose affinity chromatography were resolved by SDS-PAGE and visualized by silver staining. The control sample was prepared from GST-transfected cells. RAF-associated proteins detected by mass spectrometry are indicated with arrows. Besides 14-3-3 proteins, several heat shock proteins (HSP90, HSP70, HSP40, and Cdc37) and EF1 were identified in the RAF complexes. The same composition of the 14-3-3 proteins attached to RAF kinases has been observed in three independent measurements using different RAF preparations. MW, molecular weight. B, mammalian 14-3-3 proteins associate in vitro with A-, B-, and C-RAF in an isoform-specific manner (BIACore measurements). Purified and GST-tagged mammalian 14-3-3 proteins were captured by immobilized (immob.) anti-GST antibody as indicated. Approximately 1200 resonance units were bound for each binding assay. In the next step his-tagged A-, B-, or C-RAF was injected, and the association-dissociation curves were monitored as illustrated in the supplemental Fig. S1. To compare the binding specificity between the different 14-3-3/RAF combinations, the amounts of specific binding of RAF to 14-3-3 (unspecific binding of RAF to GST was subtracted) were summarized. C, heterodimeric forms of 14-3-3 proteins reveal similar binding properties compared with homodimeric forms (see supplemental Fig. S1). In this assay GST-C-RAF was first immobilized to the biosensor chip. Next, His-tagged 14-3-3 proteins purified from SF9 insect cells including yeast 14-3-3 protein BMH1 were injected as indicated. The binding conditions were the same as described in B. The purity of 14-3-3 samples used in the binding assays presented in C was documented by SDS-PAGE and Coomassie Blue staining (see inset in C).
Regulation of RAF Activity by 14-3-3 Proteins

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 isoform-specific manner. The finding that B-RAF, in contrast to C-RAF, associates with numerous 14-3-3 isoforms (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 proteins regarding association with RAF we isolated from S9 insect cells, a His-tagged 14-3-3 heterodimer consisting of 14-3-3ζ and ε (referred here as 14-3-3ζ/14-3-3ε, see Fig. 1C). In this binding experiment the GST-tagged C-RAF was immobilized to the biosensor chip and purified homo- and heterodimeric forms of 14-3-3 proteins. The extents of kinase activities presented in Fig. 2 document that B- and C-RAF expressed in yeast 14-3-3 homolog BMH1 can be considered slow dissociation of the 14-3-3ζ/14-3-3ε heterodimer may indicate a bivalent binding to C-RAF as proposed for inactive cytosolic C-RAF-14-3-3 complex (30). Again 14-3-3ε exhibited very low affinity for C-RAF comparable with data presented in Fig. 1B. Interestingly, the budding yeast 14-3-3 protein BMH1 bound with similar affinity to C-RAF compared with 14-3-3ζ. To ascertain whether the 14-3-3 samples used in the binding assay presented in Fig. 1C exist indeed in dimeric form, we performed size exclusion chromatography using an AKTA system (see "Experimental Procedures"). All 14-3-3 proteins depicted in Fig. 1C appeared at ~70 kDa, indicating dimer formation (data not shown). Together, results presented here indicate that regarding RAF activation, the relative affinity differences between 14-3-3 heterodimers versus homodimers for RAF binding may not be the decisive factors in vivo. Rather, the composition of the 14-3-3 dimers and the structure of the 14-3-3 binding domains in the client proteins may be critical for the efficiency and specificity of 14-3-3 coupling.

RAF Kinases Associate in Vivo Functionally with Both Hetero- and Homodimeric Forms of 14-3-3 Proteins—Results presented in Fig. 1C demonstrate that C-RAF associates in vitro effectively with both homo- 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).

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 14-3-3ε.

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 (C-RAF-S259A/S621A and C-RAF-S729A), as well as mutants impaired in both 14-3-3 coupling domains (C-RAF-S259A/S621A and B-RAF-S365A/S729A). Importantly, data presented in Fig. 2 document that B- and C-RAF expressed in yeast associated effectively with both hetero- and homodimeric forms of yeast 14-3-3 proteins. The extents of kinase activities were comparable with each other regardless of whether only BMH1 or BMH1-BMH2 complex was present in the yeast cells. Substitution of serines at the internal or C-terminal binding domain did not considerably influence the activation of B-RAF. Contrary to this finding, mutation of serine 621 prevented activation of C-RAF (Fig. 2B). The results obtained using yeast as a model system support our in vitro measurements shown in Fig. 1 and supplemental Fig. S1 and corroborate the validity and physiological relevance of such binding assays performed with recombinant proteins.

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 isoforms bind differentially to RAF isoforms. However, these data provide no information about the binding strength of 14-3-3 to the indi-
individual 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^{-8} M were mixed with RAF proteins, and the degree of RAF/14-3-3 interaction 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 IC_{50} 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 (RSAPSEP) of RAF kinases is highly conserved, the sequence surrounding serine 365 in B-RAF (RSSpSAP) 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.

Regarding kinase activities of these C-RAF mutants, the substitution of serine by alanine in position 621 (C-RAF-S259A) 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 by alanine in position 621 (C-RAF-S621A) 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.
Regulation of RAF Activity by 14-3-3 Proteins

A

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FIGURE 4. Analysis of the phosphorylation state of 14-3-3 binding sites in A-, B-, and C-RAF and changes in 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, immunoprecipitation; 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
Regulation of RAF Activity by 14-3-3 Proteins

A

**C-RAF:** wild type S621A S259A

![Graph showing C-RAF binding to 14-3-3 proteins](image)

B

**C-RAF binding to 14-3-3 isozymes in the presence of PHB**

![Graph showing C-RAF binding to 14-3-3 isozymes](image)

C

**GFP-C-RAF**

![Graph showing PHB binding](image)

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**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-
Regulation of RAF Activity by 14-3-3 Proteins

ated 14-3-3 proteins goes 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.

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-

4 E. Nekhoroshkova S. Albert, M. Becker, and UR. Rapp manuscript in revision.
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. These data support our working hypothesis illustrated in Fig. 2B, according to which PHB associates tightly with a C-RAF mutant 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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Regulation of RAF Activity by 14-3-3 Proteins: RAF KINASES ASSOCIATE FUNCTIONALLY WITH BOTH HOMO- AND HETERODIMERIC FORMS OF 14-3-3 PROTEINS

Andreas Fischer, Angela Baljuls, Joerg Reinders, Elena Nekhoroshkova, Claudia Sibilski, Renate Metz, Stefan Albert, Krishnaraj Rajalingam, Mirko Hekman and Ulf R. Rapp

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