Unique N-region Determines Low Basal Activity and Limited Inducibility of A-RAF Kinase

THE ROLE OF N-REGION IN THE EVOLUTIONARY DIVERGENCE OF RAF KINASE FUNCTION IN VERTEBRATES

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In mammals the RAF family of serine/threonine kinases consists of three members, A-, B-, and C-RAF. A prominent feature of RAF isoforms regards differences in basal and inducible kinase activities. To elucidate the nature of these differences, we studied the role of the nonconserved residues within the N-region (Negative-charge regulatory region). The nonconserved amino acids in positions $-3$ and $+1$ relative to the highly conserved serine 299 in A-RAF and serine 338 in C-RAF have so far not been considered as regulatory residues. Here we demonstrate the essential role of these residues in the RAF activation process. Substitution of tyrosine 296 in A-RAF to arginine led to a constitutively active kinase. In contrast, substitution of glycine 300 by serine (mimicking B- and C-RAF) acts in an inhibitory manner. Consistent with these data, the introduction of glycine in the analogous position of C-RAF (S339G mutant) led to a constitutively active C-RAF kinase. Based on the three-dimensional structure of the catalytic domain of B-RAF and using the sequences of the N-regions of A- and C-RAF, we searched by molecular modeling for the putative contact points between these two moieties. A tight interaction between the N-region residue serine 339 of C-RAF and arginine 398 of the catalytic domain was identified and proposed to inhibit the kinase activity of RAF proteins, because abrogation of this interaction contributes to RAF activation. Furthermore, tyrosine 296 in A-RAF favors a spatial orientation of the N-region segment, which enables a tighter contact to the catalytic domain, whereas a glutamine residue at this position in C-RAF abrogates this interaction. Considering this observation, we suggest that tyrosine 296, which is unique for A-RAF, is a major determinant of the low activating potency of this RAF isoform.

RAF kinases are signal-integrating enzymes that possess the ability to switch tyrosine kinase signaling to serine/threonine phosphorylation and connect growth factor receptors with transcription factors. Members of the RAF protein family are present in multicellular organisms as diverse as Arabidopsis (CTR1) (1), Caenorhabditis elegans (Lin-45) (2), Drosophila (D-RAF) (3), and man (4). Unlike lower eukaryotes, which express only one RAF gene, vertebrates contain at least three members of the RAF family of proto-oncogenes, A-RAF, B-RAF, and C-RAF (4–7). Although all RAF isoforms share a high degree of sequence similarity, they are differentially regulated and may have unique functions (8–10). Historically, the best characterized RAF isoform is C-RAF. B-RAF is probably the oldest RAF kinase and the isoform most frequently activated by mutations in human cancer (8). In its nucleotide sequence B-RAF is more closely related to all other eukaryotic RAF homologs than either A-RAF or C-RAF (8, 9).

Despite intensive investigations, the mechanism of RAF kinase activation is not completely understood. Although direct experimental support is missing, it is commonly assumed that the N-terminal regulatory part of RAF interacts in unstimulated cells with the catalytic domain, promoting a closed conformation of the kinase. Association with 14-3-3 proteins may further stabilize this inactive conformation (8, 9). A current model suggests that RAF association with plasma membrane lipids or lipid rafts represents an important step in the RAF activation process (11, 12). Subsequent binding to the activated small G protein Ras (Ras-GTP) occurs in the plane of the inner leaflet of the plasma membrane. Binding to activated Ras reorients RAF molecules and induces conformational changes, which allow further activation steps such as phosphorylation events, re-association with 14-3-3 proteins, and finally heterodimerization with B-RAF (8, 13–16).

RAF kinases are composed of three domains CR1, CR2, and CR3, which are highly conserved across all RAF isoforms and species. CR1 contains a Ras binding domain (RBD) and a zinc binding domain, also called cysteine-rich domain. The serine/threonine-rich CR2 domain contains the inhibitory Ser-259 phosphorylation site that is highly regulated and that serves as...
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an internal binding site for 14-3-3 protein (8). Phosphorylation of this region and various protein-protein interactions via CR2 affect the localization and activation of RAF isoforms (17, 18). The C-terminal CR3 is the catalytic kinase domain of RAF proteins and is also subject to regulation by phosphorylation of conserved residues Thr-491 and Ser-494 in the activation loop in the case of C-RAF. These residues are homologous to Thr-452 and Thr-455 of A-RAF, as well as to Thr-599 and Ser-602 of B-RAF, where mutation of these amino acids to alanine results in resistance to growth factor activation, whereas substitution by glutamic acid results in constitutive B-RAF activity independent of activated Ras (19). Similarly, mutation of the corresponding residues in C-RAF influences kinase activity (20). However, the importance of these conserved residues for A-RAF activation has not yet been evaluated. Recently, Ser-471 of C-RAF and the corresponding residue of B-RAF (Ser-578) were identified as novel in vivo RAF phosphorylation sites. Mutation analysis demonstrated that phosphorylation of these sites is critical for RAF kinase activity and for its interaction with mitogen-activated protein kinase kinase (MEK) (21).

Additional phosphorylation sites in C-RAF at positions Ser-43 and Ser-621 were identified originally by Morrison et al. (17). Whereas the phosphorylated serine in position 621 represents a binding site for 14-3-3 proteins (13, 22, 23), the role of Ser-43 phosphorylation in the regulation of RAF kinase is not fully understood. Furthermore, a short conserved sequence in front of the kinase domain, also called N-region (the name is derived from Negative-charge regulatory region), has been reported to be necessary for the basal activity and growth factor-induced activation of RAF kinases. This region contains one highly conserved serine, which is present in all three RAF isoforms (Ser-299 in A-RAF, Ser-338 in C-RAF, and Ser-446 in B-RAF) and two conserved tyrosines (Tyr-301/Tyr-302 in A-RAF and Tyr-340/Tyr-341 in C-RAF) at positions where in B-RAF aspartates are located (Asp-448/Asp-449). Stimulation-dependent phosphorylation of these sites positively regulates kinase activity of C- and B-RAF (24–27). In the case of A-RAF, a stimulating role of tyrosines 301/302, corresponding to tyrosines 340/341 in C-RAF, has been demonstrated (26), whereas involvement of serine 299 in regulation of A-RAF kinase activity has not been investigated.

Mammalian RAF isoforms show remarkable differences with respect to basal and growth factor-induced activity. B-RAF displays extraordinarily high basal kinase activity compared with C-RAF, is weakly responsive to oncogenic Ras, and is not stimulated at all by activated SPC (26). In contrast, C-RAF possesses low activity in nonstimulated cells but can be readily activated by oncogenic Ras and SPC (24–27). Although A-RAF has also been shown to be activated by Ras and SPC, the level of A-RAF activity obtained under these conditions is only ~20% of that for C-RAF and is considerably lower compared with B-RAF (26).

To elucidate the origin of these dramatic differences in regulation of RAF isoforms, we have studied the functional contribution of the nonconserved residues within the N-region. The nonconserved amino acids in positions −3 and +1 relative to the highly conserved serine 299 in A-RAF and serine 338 in C-RAF have so far not been considered as regulatory residues. Here we demonstrate an essential role of these residues in the course of the RAF activation process. Substitution of tyrosine at position 296 in A-RAF to arginine led to a constitutively active RAF kinase. In contrast, substitution of glycine at position 300 by serine (mimicking B- and C-RAF) acts in an inhibitory manner, preventing Ras-dependent activation of A-RAF. Consistently, with these data the introduction of glycine in the analogous position of C-RAF (S339G mutant) led to a constitutively active C-RAF kinase. Using the crystal structure of the catalytic domain of B-RAF (CR3) and the amino acid sequence of the N-regions of RAF isoforms, we searched by molecular modeling for the putative contact points between these two moieties. Based on the in silico results of the molecular modeling and on in vivo experimental data, we postulate that the tight association between N-region and the catalytic domain negatively regulates RAF kinase activity. The data presented here fit to an evolutionary model where A-RAF derives from C-RAF via mutations that down-regulate basal activity and rescue inducibility at low level.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and T4 DNA ligase were obtained from Bio-Lab. The Phusion DNA polymerase was from Finnzymes. Proteinase inhibitors, leupeptin, aprotinin, antipain, and phenylmethylsulfonyl fluoride were obtained from Sigma. Nonidet P-40 was from Roche Applied Science. Anti-phospho-ERK, anti-Myc, anti-ERK, anti-actin, and anti-Lck antibodies were from Santa Cruz Biotechnology. Antibody against H-Ras was from BD Transduction Laboratories. Phosphospecific antibodies directed against Ser(P)-338 and Tyr(P)-340/341 of C-RAF were from Upstate Biotechnology, Inc., and BioSource. Horseradish peroxidase-conjugated polyclonal anti-rabbit and anti-mouse IgGs were obtained from Dianova. Anti-M2P2K was from Scheko Biotech (kindly provided by S. Mazurek). Anti-KDEL and anti-PARP antibody were obtained from StressGen and Biomer, respectively. Antibody against vimentin was from Dako (kindly provided by R. Houben).

DNA Construction—Cloning of C-terminal Myc-tagged human A- and C-RAF was performed as follow. A-RAF and C-RAF cDNAs were amplified by PCR. In the case of A-RAF the upstream primer sequence was 5’-AGGGAAAAAGGGGCCGCAATGGAGCCACCCGAGGGGC-3’, which contained a NotI restriction site (underlined). The downstream primer sequence was 5’-TCCCGCGAGCCGAACAGGGGGCTGC-3’, which contained a SacII restriction site (underlined). For C-RAF the upstream primer sequence was 5’-AGGAAAAAGGGGCCGCAATGGAGCCACCCGAGGGGC-3’, containing a NotI restriction site (underlined). The downstream primer sequence with a SacII restriction site (underlined). The amplified cDNAs and the mammalian expression vector pcDNA3.1/myc-His B (Invitrogen) were cut with NotI and SacII enzymes and, subsequently, ligated by use of T4 DNA ligase to give the expression plasmids for C-terminal Myc-tagged A- and C-RAF. The site-specific mutations in the N-region of A- and C-RAF (listed in Fig. 1B) were introduced using QuikChange site-directed mutagenesis kit (Stratagene) according to the manufac-
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Infection of Sf9 Insect Cells—For the immunoprecipitation of recombinant A-RAF proteins, 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 buffer and pelleted at 1100 rpm (Megafuge 1.0R, Heraeus). The Sf9 pellets were lysed in 2 ml of Nonidet P-40 lysis buffer containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 25 mM NaF, 10% (v/v) glycerol, 1 mM Na3VO4, 0.75% Nonidet P-40, and a mixture of standard proteinase inhibitors (1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) for 45 min with gentle rotation at 4 °C. The lysates were centrifuged at 27,000 × g (SS34 rotor, Sorvall centrifuge) for 30 min at 4 °C. The supernatants were used for anti-A-RAF immunoprecipitation.

Cell Culture, Transfection, and Immunoprecipitation—COS7 and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin at 37 °C, in humidified air with 5% CO2. cDNAs encoding the Myc-tagged A-RAF and C-RAF variants were transfected either alone or with H-Ras12V and Lck into COS7 or HEK293 cells using jetPEI transfection reagent (Biomol); a total of 10 µg of recombinant DNA per 10-cm Petri dish was used. 24 h after transfection the cells were serum-starved by placement in Dulbecco’s modified Eagle’s medium containing 0.1% fetal calf serum, 2 mM Hepes, pH 7.6, 150 mM NaCl, 25 mM β-mercaptoethanol, 0 mM sodium pyrophosphate, 1 mM Na3VO4, 25 mM NaF, 1% Nonidet P-40, and proteinase inhibitors (see above) for 45 min at 4 °C. In some experiments cells were incubated with 100 ng/ml EGF for 1 or 5 min before lysis. All lysates were clarified by centrifugation at 27,000 × g for 15 min and incubated for 1 h at 4 °C with anti-Myc antibody. After addition of protein G-agarose (20-μl bead volume), the incubation was continued at 4 °C for 2 h. The agarose beads were washed twice with lysis buffer containing 0.2% Nonidet P-40 and once with kinase assay buffer. Kinase assay was carried out directly with immunoprecipitated proteins as described below.

In Vitro RAF Kinase Assay—Kinase assays were performed using recombinant MEK and ERK-2 as substrates in 25 mM Hapes, pH 7.6, 150 mM NaCl, 25 mM β-glycerophosphate, 10 mM MgCl2, 1 mM dithiothreitol, 1 mM Na3VO4, and 500 µM ATP buffer (50 µl final volume). Following the addition of RAF-containing samples, the reaction mixtures were incubated for 30 min at 30 °C. The kinase assay mixtures were then supplemented with Laemmli buffer, boiled for 5 min at 100 °C, and applied to SDS-PAGE. After Western blotting the extent of ERK phosphorylation was determined by anti-phospho-ERK antibodies and enhanced chemiluminescence (ECL, Amer sham Biosciences).

Subcellular Fractionation—Cell fractions were isolated using the ProteoExtract subcellular proteome extraction kit (Calbiochem). COS7 were grown on the 10-cm Petri dishes, transfected with cDNA encoding Myc-tagged C-RAF variants, and serum-starved as already described above. The cells were fractionated into four subproteomic fractions (cytoplasmic and nuclear fractions, fractions of whole membranes, and cytoskeleton) according to the manufacturer’s protocol. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membrane. The recombinant C-RAF proteins were detected by anti-Myc antibody. The selectivity of subcellular extraction was documented by immunoblotting against marker proteins (M2PK for cytosolic fraction, KDEL for membrane fraction, PARP for nuclear fraction, and vimentin for cytoskeletal fraction). RAF kinase assays were performed with the samples of each fraction as described above.

Modeling of the N-region Interactions with the Catalytic Part of RAF Kinases—The structure models for the kinase domain of A-RAF and C-RAF were obtained by homology modeling using the crystal structure of B-RAF catalytic domain as a template. The amino acid sequences of all three homologs were aligned using ClustalW, and the differing amino acid residues of B-RAF with respect to A- and C-RAF were exchanged using the software Quanta2005 (Accelrys Inc.). Side chain orientations of the substituted residues were first optimized by the tool X-Build in Quanta2005 to yield side chain orientations that are most common for the particular amino acid type unless the side chain orientation resulted in a steric clash with neighboring residues.

The N-terminal extension, which is not present in the crystal structure of the B-RAF template (residues Gly-295 to Gly-300 in A-RAF and residues Gly-334 to Ser-339 in C-RAF), was first built with an extended β-strand conformation for the backbone atoms. Backbone and side chain atoms of the N-terminal extension were then optimized by energy minimization using 500 steps of AdoptedRaphson Newton algorithm and the Charmm22 force field employing only geometrical energy terms and no electrostatics. As the final step, a full energy term minimization was performed for the N-terminal extension, and all other residues were kept fixed, employing a distance-dependent dielectric treatment for the electrostatics.

RESULTS

Phosphorylation of the Conserved Serine 299 (A-RAF), 338 (C-RAF), 446 (B-RAF) in the N-region Is Predicted to Depend on Amino Acids at Position −3—Because it has been observed that the level of A-RAF activity following growth factor stimulation is considerably lower compared with C- and B-RAF (26) and because there are major differences in the sequence of the regulatory N-region, we decided to explore the role of nonconserved amino acids of this region. We noticed that serine 339 of C-RAF (the homologous residue in B-RAF is Ser-447), which is next to the strongly conserved serine 338, is substituted by glycine in the case of A-RAF (Gly-300) (Fig. 1A). Additionally, the amino acid in position 296 in A-RAF (tyrosine 296), which is analogous to glutamine 335 in C-RAF and to arginine 443 in B-RAF, is variable for all of three isoforms.

In silico analysis of the phosphorylation efficiency by using the NetPhos 2.0 algorithm (28) revealed that the phosphorylation efficiency of the conserved serine 299 (A-RAF), 338 (C-RAF), and 446 (B-RAF) differed enormously between RAF
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A. B-RAF

C-RAF

A-RAF

\[
\begin{array}{c}
\text{B-RAF} \\
\text{N} \\
\text{CR1} \\
\text{CR2} \\
\text{CR3} \\
\text{C} \\
442 \\
434 \\
295 \\
\end{array}
\]

\[
\begin{array}{c}
\text{C-RAF} \\
\text{N} \\
\text{RB} \\
\text{CR1} \\
\text{CR2} \\
\text{CR3} \\
\text{C} \\
259 \\
259 \\
214 \\
\end{array}
\]

\[
\begin{array}{c}
\text{A-RAF} \\
\text{N} \\
\text{RB} \\
\text{CR1} \\
\text{CR2} \\
\text{CR3} \\
\text{C} \\
229 \\
279 \\
362 \\
\end{array}
\]

B. A-RAF-Y296R, A-RAF-G300S, A-RAF-Y296R/G300S, A-RAF-Y296R/Y301D/Y302D, C-RAF-G335Y, C-RAF-G335R, C-RAF-S339G, C-RAF-G335Y/S339G, C-RAF-G335Y/Y341D

\[
\begin{array}{c}
\text{A-RAF-Y296R} \\
\text{A-RAF-G300S} \\
\text{A-RAF-Y296R/G300S} \\
\text{A-RAF-Y296R/Y301D/Y302D} \\
\text{C-RAF-G335Y} \\
\text{C-RAF-G335R} \\
\text{C-RAF-S339G} \\
\text{C-RAF-G335Y/S339G} \\
\text{C-RAF-G335Y/Y341D} \\
\end{array}
\]

FIGURE 1. Schematic presentation of A-, B-, and C-RAF proteins and sequences of the N-region of wild type proteins and of the A- and C-RAF substitution mutants investigated in this study. A, upper panel, schematic presentation of A-, B-, and C-RAF kinases with conserved regulatory phosphorylation sites within N-region and within 14-3-3 protein-binding sites. Light shaded boxes, nonconserved parts of the sequence; dark shaded boxes, conserved regions. CR1 consists of the Ras binding domain (RBD) and the cysteine-rich domain (CR1). CR2 is the serine-threonine rich domain, and CR3 is the kinase domain. Phosphorylation sites within the N-region are indicated by a red rectangle. Lower panel, alignment of the N-region sequences of three RAF isoforms. The variable amino acids are indicated by colored rectangles. B, alignment of the N-region sequences of A- and C-RAF substitution mutants investigated in this study. Green rectangles, substitutions at position –3 to conserved phosphoserine; purple rectangles, substitutions at position +1 to conserved phosphoserine; orange rectangles, substitutions at positions +2 and +3 to conserved phosphoserine.

isomers. According to the prediction, phosphorylation efficiency of serine 338 in C-RAF and serine 446 in B-RAF is 98.1 and 99.8%, respectively, whereas the phosphorylation probability of the homologous serine (Ser-299) in A-RAF is strongly reduced to 15.3%. Moreover, phosphorylation efficiency of the conserved serine 299 in A-RAF is predicted to depend largely on the nature of the amino acid at position –3 (Fig. 1A). Thus, the phosphorylation probability of serine 299 in A-RAF increases up to 76.7% if tyrosine at position –3 is substituted by glutamine (corresponding to C-RAF), and it further increases to 99.1% if the residue in position –3 is an arginine (as in B-RAF). In the case of C-RAF, the effect of the amino acid at the position –3 on phosphorylation efficiency of the conserved serine 338 is moderate. Substitution of glutamine 335 by tyrosine (mimicking A-RAF) is predicted to decrease the phosphorylation efficiency of serine 338 to 80.7%. In contrast, the degree of phosphorylation of the conserved serine 446 in B-RAF does not seem to be affected by amino acid in the position –3 relative to phosphorylation site. However, if the aspartates 448 and 449 are substituted by tyrosines (thus, mimicking A- and C-RAF), the phosphorylation efficiency of the serine 446 in B-RAF becomes highly dependent on the nature of the amino acid in position –3, suggesting that aspartates in positions +2 and +3 may abolish the dependence of serine 446 phosphorylation on amino acid in position –3.

On the other hand, the variable amino acid Gly-300 in A-RAF, Ser-339 in C-RAF, and Ser-447 in B-RAF at the position +1 relative to the implicated serine residue was predicted to have no significant effect on the phosphorylation of conserved serine in all three RAF isoforms. According to the prediction by NetPhos 2.0, serine 339 in C-RAF and the homologous serine 447 in B-RAF represent likely phosphorylation sites (99.5 and 98.8% phosphorylation probability, respectively). However, at present quantitative data regarding phosphorylation of these residues in C- and B-RAF are not available, and in vivo phosphorylation of these sites was reported to have no effect on RAF activation (27). Notably, this conserved phosphorylation site is absent in A-RAF because there is a glycine (Gly-300) instead of a serine (Fig. 1A).

Following the results of NetPhos2.0 analysis, we speculated that the amino acid at position –3 relative to the conserved serine may critically influence phosphorylation of this serine and thus may contribute to regulation of RAF kinase activity.

Mutation in the N-region Leads to a Constitutively Active Form of A-RAF Kinase—Considering the prediction data and the reports that phosphorylation of the conserved serine 338 in C-RAF and serine 446 in B-RAF is one of the most important events in the process of kinase activation, we hypothesized that the predicted moderate degree of phosphorylation of homologous serine 299 in A-RAF (possibly caused by the presence of tyrosine in the position 296) may be responsible for the weak kinase activity of this isoform. To examine this possibility, we generated an A-RAF substitution mutant, in which tyrosine 296 was replaced by arginine (Fig. 1B, A-RAF-Y296R). In addition, because NetPhos2.0 did not provide further information on the involvement of the glycine residue at position 300 in phosphorylation of serine 299 in A-RAF, we decided to test the contribution of Gly-300 to A-RAF kinase activation by mutagenesis. For this purpose we generated A-RAF substitution mutants A-RAF-G300S and A-RAF-Y296R/G300S, the latter partially resembling the N-region sequence of B-RAF. We also prepared A-RAF mutants, where the tyrosines 301 and 302 were substituted by aspartates (A-RAF-Y301D/Y302D and A-RAF-Y296R/G300S/Y301D/Y302D, designated as A-RAF-DD and A-RAF-Y296R/G300S/DD, respectively) to investigate an N-region structure, completely homologous to B-RAF (Fig. 1B).

All mutants carried a C-terminal Myc tag. Because A-RAF appeared not to be responsive to EGF stimulation alone in
HEK293 cells (data not shown), we decided to use the combination of H-Ras12V and Lck, which has been reported to be a potent stimulus for activation of A-RAF kinase (26). Upon transfection of each A-RAF construct into HEK293 cells either alone or together with H-Ras12V/Lck, A-RAF proteins were immunoprecipitated by means of an anti-Myc antibody and subjected to in vitro coupled kinase assay. The extent of A-RAF kinase activity was monitored by anti-phospho-ERK antibody as described under "Experimental Procedures".

As shown in Fig. 2, A-RAF wild type as well as A-RAF substitution mutants A-RAF-Y296R, A-RAF-G300S, and A-RAF-Y296R/G300S are highly responsive to stimulation by oncogenic Ras and Lck. Although wild type A-RAF isolated from starved HEK293 cells was completely inactive, co-transfection with H-Ras12V and Lck resulted in elevated kinase activity. The same effect was also observed with the A-RAF-G300S mutant. Substitution of A-RAF tyrosine 296 by arginine (A-RAF-Y296R mutant) led to elevated kinase activity even in the absence of any stimulatory agents, demonstrating that this mutant is constitutively active (Fig. 2). Even more strikingly, the elevated basal activity of the A-RAF-Y296R mutant was almost completely abrogated by the additional introduction of a serine residue instead of glycine at position 300 in the A-RAF-Y296R/G300S double mutant (Fig. 2), suggesting that serine at this position may act in an inhibitory fashion with respect to basal A-RAF kinase activity. Because substitution of tyrosines 301 and 302 by aspartates in A-RAF generates a kinase that can be activated by oncogenic Ras, but not by Lck (26), we stimulated the A-RAF-Y296R/G300S/DD mutant with H-Ras12V only. Fig. 2 shows that this mutant had an elevated basal level of activity and was still responsive to stimulation by oncogenic Ras, indicating that the inhibitory effects of G300S substitution did not significantly impair the activating potential of Y301D/Y302D substitution.

Because we did not observe significant differences in kinase activity of A-RAF mutants following co-transfection with H-Ras12V/Lck (see Fig. 2), we attempted to improve discrimination by activating with H-Ras12V only. To exclude differences in kinase activity of A-RAF constructs as a consequence of transfection variability, the HEK293 cells were first transfected with H-Ras12V. 48 h after transfection the cells were transfected again with plasmids expressing each A-RAF construct. 12 h after the second transfection the cells were starved for an additional 12 h. A-RAF activity was subsequently monitored using anti-Myc A-RAF immunoprecipitation and MEK/ERK-coupled kinase assay.

Following activation by oncogenic Ras, the A-RAF-Y296R mutant revealed considerably higher kinase activity compared with A-RAF WT (Fig. 3, A and B). This observation is in line with the results obtained with the same mutant under nonactivating conditions (see Fig. 2). Surprisingly, the G300S substitution, mimicking B- and C-RAF in this position, completely abrogated the activation by Ras12V resulting in an inactive kinase (Fig. 3, A and B). A similar effect was also observed with the A-RAF-Y296R/G300S mutant, indicating that serine in position 300 of A-RAF indeed acts inhibitory under stimulating (see Fig. 3, A and B) as well as nonstimulating (see Fig. 2) conditions. Furthermore, kinase activity of the A-RAF-Y296R/G300S/DD mutant was slightly lower than that of the A-RAF-DD variant, suggesting a dominant role of serine at position 300 over the otherwise activating tyrosine 296 to arginine exchange (Fig. 3, A and B).

In agreement with the NetPhos2.0 prediction, activation of A-RAF mutants was associated with phosphorylation of serine 299 (C-RAF Ser-338 homolog), as judged by reactivity with the anti-C-RAF-Ser(P)-338 antibody. The antibody recognizes the RDSS motif in both C- and B-RAF, as well as in A-RAF mutants carrying the G300S substitution (Fig. 3, A and C). Whereas the A-RAF-Y296R/G300S double mutant was slightly phosphorylated, the single A-RAF-G300S mutant was not phosphorylated at all (see Fig. 3, A and C).

To determine whether phosphorylation of serine 299 is essential for A-RAF activation as shown for serine 338 in C-RAF (25), we generated the A-RAF-S299A mutant. Wild type and mutant A-RAF expressed in Sf9 cells alone or in combination with H-Ras12V and/or with Lck were tested for kinase activity and the level of tyrosine phosphorylation at position 301/302. As shown in supplemental Fig. S1, we observed enhanced tyrosine phosphorylation in the presence of Lck in both A-RAF wild type and A-RAF-S299A mutant, suggesting that S299A substitution did not prevent the tyrosine phosphorylation in A-RAF-S299A. Importantly, this substitution almost completely abrogated activation of A-RAF kinase. Thus, our data suggest that the residue serine 299 is essential for A-RAF kinase activity, and substitution of this residue with alanine prevents activation of A-RAF by both oncogenic Ras and Lck. Moreover, our experimental results demonstrate that tyrosine

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**FIGURE 2.** A-RAF-Y296R mutant is constitutively active in starved cells. C-terminally Myc-tagged A-RAF wild type (WT) and A-RAF substitution mutants (G300S, Y296R, Y296R/G300S, and Y296R/G300S/DD) were expressed in HEK293 cells in the presence and absence of H-Ras12V and Lck. The cells were serum-starved for the last 12 h of incubation and lysed using detergent containing buffer. Levels of A-RAF in each sample were determined and balanced against each other, so that equivalent amounts of A-RAF were immunoprecipitated (IP) by anti-Myc antibody. Subsequently, coupled kinase assay using MEK and ERK as substrates has been performed, and the extents of kinase activities were monitored by anti-pERK antibody. Expression efficiency of A-RAF mutants was determined and balanced against each other, so that equivalent amounts of A-RAF were immunoprecipitated (IP) by anti-Myc antibody. Subsequently, coupled kinase assay using MEK and ERK as substrates has been performed, and the extents of kinase activities were monitored by anti-pERK antibody. Expression efficiency of A-RAF mutants was determined and balanced against each other, so that equivalent amounts of A-RAF were immunoprecipitated (IP) by anti-Myc antibody. Subsequently, coupled kinase assay using MEK and ERK as substrates has been performed, and the extents of kinase activities were monitored by anti-pERK antibody.
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**A.** A-RAF: WT, G300S, Y296R, Y296R/DD

α-pERK IB
α-ERK IB
α-pS338 IB
α-Myc IB
α-H-Ras IB
α-Actin IB

**B.** A-RAF kinase activity

| Protein | WT | G300S | Y296R | Y296R/DD |
|---------|----|-------|-------|---------|
| A-RAF   | 1  | 1     | 1     | 1       |
| A-RAF(Y296R) | 2 | 2     | 2     | 2       |
| A-RAF(Y296R/G300S) | 3 | 3     | 3     | 3       |

**C.** In vitro kinase assay

**D.** Serine 299 phosphorylation

| Protein | WT | G300S | Y296R | Y296R/DD |
|---------|----|-------|-------|---------|
| A-RAF   | 1  | 1     | 1     | 1       |
| A-RAF(Y296R) | 2 | 2     | 2     | 2       |
| A-RAF(Y296R/G300S) | 3 | 3     | 3     | 3       |

**FIGURE 3.** Y296R and G300S substitutions affect A-RAF activation by H-Ras12V. A, Western blot analysis of A-RAF kinase activity and serine 299 phosphorylation. HEK293 cells were first transfected with H-Ras12V only, then divided, and secondary transfected with A-RAF wild type (WT) or with A-RAF substitution mutants (G300S, Y296R, Y296R/G300S, Y296R/G300S/DD, and DD) carrying C-terminal Myc tag. After the cells have been lysed, levels of A-RAF in each sample were determined and balanced against each other, so that equivalent amounts of A-RAF were immunoprecipitated (IP) by anti-Myc antibody. Subsequently, coupled kinase assay using MEK and ERK as substrates has been carried out, and the extents of kinase activities were monitored by anti-pERK antibody. Expression efficiency of H-Ras12V was determined using an anti-H-Ras antibody. Actin immunodetection was used as a loading control. IB, immunoblot. B, quantification of A-RAF kinase activity. Representative blots from A were quantified by optical densitometry. The quantification results are expressed in terms of fold activation, where 1-fold of activity represents the amount of activity determined for A-RAF wild type. C, quantification of A-RAF phosphorylation on serine 299 by use of anti-C-RAF-Ser(P)-338 antibody. Representative blots from A were quantified by optical densitometry. The quantification results are expressed in terms of fold phosphorylation, where 1-fold of phosphorylation represents the amount of phosphate incorporation determined for A-RAF-Y296R/G300S mutant. D, tyrosine phosphorylation of A-RAF wild type and A-RAF mutants. HEK293 cells were transfected with C-terminally Myc-tagged A-RAF wild type or with the same A-RAF substitution mutants as shown in A in the presence and absence of H-Ras12V or in the presence of both H-Ras12V and Lck. The cells were serum-starved for the last 12 h of incubation and lysed using detergent-containing buffer. A-RAF proteins were immunoprecipitated by anti-Myc antibody, and the extent of tyrosine phosphorylation was monitored by use of the anti-phosphotyrosine antibody 4G10. A-RAF-DD and A-RAF-Y296R/G300S-DD mutants are not shown, because they revealed only marginal tyrosine phosphorylation because of the substitution of Tyr-301 and Tyr-302 by asparagines.

Phosphorylation does not compensate for the serine 299 to alanine mutation with respect to kinase activity of A-RAF.

Unfortunately, the anti-Ser(P)-338 antibody directed against the N-region of C-RAF could not be used for detection of phosphorylation at position 299 of A-RAF WT and A-RAF mutants containing glycine at position 300, such as A-RAF-Y296R, presumably because of the differences in the structure of the recognition sequence. On the other hand, we conclude that elevated activity of the constitutively active A-RAF-Y296R mutant was not caused by phosphorylation of tyrosines 301/302, because we did not detect any tyrosine phosphorylation using anti-phosphotyrosine antibody 4G10 in the basal state or following stimulation with H-Ras12V in A-RAF wild type as well as in A-RAF-Y296R, A-RAF-G300S, and A-RAF-Y296R/G300S mutants. In contrast, in the presence of H-Ras12V and Lck considerable tyrosine phosphorylation was detected in all A-RAF mutants (Fig. 3D).

To address the question, whether the extent of kinase activity of A-RAF mutants compared with A-RAF wild type might correlate with the phosphorylation of tyrosines upon stimulation by Src family kinases alone, we transfected the cells with A-RAF constructs in the presence or absence of Lck. As shown in supplemental Fig. S2, the pattern and the extent of kinase activities of A-RAF mutants were comparable under both non-stimulating conditions and upon co-transfection with Lck (the slight increase in kinase activity of some samples in the presence of Lck is because of the higher amounts of A-RAF proteins). In both cases the level of kinase activity of the A-RAF-Y296R mutant was elevated, whereas kinase activity of A-RAF mutants carrying G300S substitution was diminished compared with wild type (see supplemental Fig. S2A). Importantly, the pattern of tyrosine phosphorylation did not correlate with the pattern of kinase activity of A-RAF mutants. Consistent with the Fig. 3D, we did not observe any tyrosine phosphorylation in the absence of Lck. On the other hand, upon co-transfection with Lck a moderate tyrosine phosphorylation was detectable, whereby the levels of tyrosine phosphorylation were comparable for all A-RAF mutants. We conclude that the observed differences in tyrosine phosphorylation are because of the variations in Lck expression, because in the case of equal Lck expression we did not detect differences in tyrosine phosphorylation (compare supplemental Fig. S2, A and B).

The results presented here confirm the predictions from NetPhos2.0, suggesting that amino acid at position −3 relative to the conserved serine 299 in the N-region determines the phosphorylation efficiency of this serine and consequently regulates the kinase activity of A-RAF. Furthermore, as shown in
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FIGURE 4. Substitution of serine 339 by glycine in the N-region of C-RAF leads to constitutively active kinase. C-terminally Myc-tagged C-RAF wild type (WT) and C-RAF substitution mutants (Q335Y, Q335R, S339G, and Q335Y/S339G) were expressed in COS7 cells in the presence (right panel) and absence (left panel) of H-Ras12V. After 12 h of serum starvation, cells were lysed using detergent-containing buffer. Cell lysates were subjected to immunoprecipitation (IP) with anti-Myc antibody. Subsequently, coupled kinase assay using MEK and ERK as substrates has been carried out, and extents of ERK phosphorylation were monitored by anti-pERK antibody. Phosphorylation of serine 338 was examined by use of anti-C-RAF-Ser(P)-338 antibody. Expression efficiency of H-Ras12V was determined using anti-H-Ras antibody. Actin immunodetection was used as a loading control. Notice that expression degree of the sample containing C-RAF-Q335Y and H-Ras12V was lower compared with other samples. IB, immunoblot.

Fig. 3A, substitution of tyrosines at positions 301/302 to asparagines acts stimulatory toward serine 299 phosphorylation in A-RAF upon transfection with oncogenic Ras, because phosphorylation of this serine in A-RAF-Y296R/G300S/DD was about 11-fold higher than that in A-RAF-Y296R/G300S mutant.

Taken together, these results demonstrate that amino acid variations in the short N-region change profoundly the kinase properties of A-RAF protein. In particular, we show here that the substitution of glycine 300 with serine acts in an inhibitory manner preventing Ras-dependent activation, whereas replacement of tyrosine 296 by arginine, mimicking the situation in B-RAF, leads to a constitutively active A-RAF kinase possessing elevated activity even in the absence of any activating agents. These observations indicate that the poor basal activity of A-RAF compared with B-RAF can be ascribed not only to the tyrosines at positions 301/302 but also to the tyrosine at position 296 in A-RAF.

C-RAF Behaves Similar to A-RAF; Mutations at the Positions 335 and 339 in the N-region of C-RAF Critically Modulate Kinase Activity—To address the question whether substitution of amino acids at the analogous positions within the N-region of C-RAF (Gln-335 and Ser-339) would also lead to changes in serine 338 phosphorylation and consequently to changes in the activation process, we next generated C-RAF mutants, mimicking N-region sequences of A- or B-RAF. To this end we first replaced glutamine 335 in C-RAF by either tyrosine (as present in A-RAF) or arginine mimicking B-RAF (see Fig. 1B). Additionally, following our observations that the introduction of serine instead of glycine at position 300 in A-RAF (analogous to position 339 in C-RAF) led to inhibition of the activation process, we also exchanged the serine 339 in C-RAF by glycine, to examine whether this substitution might affect C-RAF activity. Furthermore, a double mutant of C-RAF (C-RAF-Q335Y/S339G) was generated, in which both residues were replaced by amino acids that are normally present in A-RAF wild type, thus resembling the N-region of A-RAF (Fig. 1B).

First, we investigated differences of serine 338 phosphorylation and kinase activity of C-RAF constructs under nonstimulating conditions and by co-transfecting COS7 cells with H-Ras12V. For this purpose the cells were transfected either with expression vectors for C-terminally Myc-tagged C-RAF WT and C-RAF substitution mutants alone or together with oncogenic Ras. The C-RAF proteins were immunoprecipitated and subjected to a coupled kinase assay. As shown in Figs. 4 and 5, the basal kinase activities (without any stimulation of the cells) exhibited already remarkable differences. Although C-RAF WT and C-RAF-Q335Y mutant displayed very low basal activities, substitution of Gln-335 by arginine (C-RAF-Q335R) resulted in an increase of basal activity. In parallel, phosphorylation of serine 338 in the C-RAF-Q335R mutant was strongly elevated compared with C-RAF WT, indicating that arginine in this position promotes phosphorylation of serine 338 (Fig. 4 and Fig. 5, B and D). More strikingly, the introduction of glycine in the position 339 instead of serine (C-RAF-S339G mutant) led to a constitutively active C-RAF kinase, having almost 20-fold higher basal activity compared with C-RAF WT (Fig. 4 and Fig. 5, A–C). These results demonstrate clearly that the introduction of glycine in the position +1 relative to Ser-338 is stimulatory with respect to C-RAF kinase activity. On the other hand, the reverse substitution of glycine by serine in the analogous position in A-RAF (G300S) led to an inactive kinase (Fig. 3A). Interestingly, the double mutant of C-RAF (C-RAF-Q335Y/S339G) also revealed elevated basal activity (Fig. 4 and Fig. 5, A–C), indicating that the inhibitory effect of the Q335Y substitution can be compensated by the S339G mutation.

Unfortunately, the degree of phosphorylation of serine 338 in C-RAF mutants S339G and Q335Y/S339G could not be determined by anti-phosphoserine 338 antibody, because the structure of recognition sequence seems to be disrupted by introduction of glycine instead of serine. Furthermore, as demonstrated in Fig. 4, co-transfection with oncogenic Ras did
FIGURE 5. Phosphorylation of conserved serine 338 does not completely correlate with the extent of kinase activity of C-RAF upon long term activation with EGF. COS7 cells were transfected with C-RAF wild type or C-RAF mutants as indicated. Following starvation for 24 h, the cells were stimulated by EGF for 1 and 5 min. Levels of C-RAF in each sample were determined and balanced against each other, so that equivalent amounts of C-RAF were immunoprecipitated (IP) by anti-Myc antibody. Subsequently, kinase activities were measured using a coupled kinase assay in the presence of recombinant MEK and ERK proteins. ERK phosphorylation was detected by phosphospecific anti-ERK antibody. Phosphorylation of serine 338 was determined by anti-C-RAF-Ser(P)-338 antibody. A, analysis of C-RAF kinase activity under nonstimulating conditions (left panel) and upon short term stimulation (1 min) with EGF (right panel). IB, immunoblot. B, analysis of C-RAF kinase activity and serine 338 phosphorylation under nonstimulating conditions (left panel) and upon long term stimulation (5 min) with EGF (right panel). C, quantification of C-RAF kinase activity. Average values derived from the data of five independent experiments were used for quantification. Quantification results are expressed in terms of fold activation, where 1-fold of activity represents the amount of activity determined for C-RAF wild type under nonstimulating conditions. D, quantification of serine 338 phosphorylation. Representative blots from B were quantified by optical densitometry. The quantification results are expressed in terms of fold phosphorylation, where 1-fold of phosphorylation represents the amount of phosphorylation determined for C-RAF wild type under nonstimulating conditions. E, tyrosine phosphorylation of C-RAF wild type and C-RAF mutants under nonactivating conditions and following stimulation with EGF. C-RAF wild type, which was expressed in the presence of oncogenic Ras and Lck and has been shown to be highly phosphorylated on tyrosines (27), was used as a positive control for the reactivity of anti-tyrosine antibody 4G10.
not significantly change the serine 338 phosphorylation and the kinase activity pattern of C-RAF mutants. However, the levels of kinase activity of each C-RAF mutant as well as degrees of phosphorylation of C-RAF WT and C-RAF-Q335R mutant were increased upon transfection with H-Ras12V (Fig. 4), indicating that despite their increased basal activity all C-RAF mutants tested are still sensitive to stimulation by oncogenic Ras. Similar results were also observed with HEK293 cells (data not shown), suggesting that differences in activation and serine 338 phosphorylation of C-RAF mutants are not cell line specific.

To test the susceptibility to activation of C-RAF substitution mutants compared with C-RAF WT under physiological conditions, we performed short and long term stimulation of cells by EGF. Here we chose COS7 cells, because of the fact that this cell line has been reported to be more sensitive to EGF activation than HEK293 cells (29). Because EGF stimulation of C-RAF is a rapid process, reaching a maximum already after 30–60 s (13, 30), we stimulated the cells for 1 and 5 min. Immediately after stimulation with EGF, the cells were lysed, and C-RAF kinase activities were determined. As shown in Fig. 5, A and C, stimulation of COS7 cells with EGF for 1 min resulted in a similar pattern of kinase activity for C-RAF WT and C-RAF mutants, as has already been observed with nonstimulated cells and with cells co-transfected with oncogenic Ras. In contrast, after 5 min of EGF stimulation all C-RAF proteins showed similar levels of maximal kinase activity (Fig. 5, B and C), suggesting that the mutations within the N-region of C-RAF are relevant for the basal activity as well as for the initial steps of the activation process; however, they do not seem to be relevant for the maximal activation. Although the levels of kinase activity of C-RAF WT, C-RAF-Q335Y, and C-RAF-Q335R did not significantly differ upon long term stimulation (5 min) with EGF, we observed significant differences of serine 338 phosphorylation in these C-RAF mutants (Fig. 5, B–D). These discrepancies indicate that phosphorylation on serine 338 regulates kinase activity of C-RAF under starvation conditions and at the initial steps of EGF stimulation, whereas additional regulatory events promote the activating process upon prolonged stimulation. Moreover, because our results shown in Figs. 4 and 5 indicate that continuous expression of oncogenic Ras did not lead to compensation of differences in kinase activity of C-RAF mutants in contrast to prolonged EGF stimulation, we suggest that C-RAF activating events initiated by EGF do not act through the H-Ras pathway alone. Interestingly, these additional activating pathways did not regulate kinase activity of C-RAF by phosphorylation of activating tyrosines in the positions 340/341, because Western blot analysis of immunoprecipitated proteins did not show any tyrosine phosphorylation following stimulation with EGF (see Fig. 5E).

Collectively, these results, together with results obtained for A-RAF, indicate that the residues in positions −3 and +1 relative to Ser-338 in C-RAF and Ser-299 in A-RAF play an important role in the activation process of these isoforms. Introduction of glycine in position +1 leads to a constitutively active C-RAF kinase, whereas substitution of glutamine in position −3 by arginine provided a mixed effect; although phosphorylation of serine 338 was strongly increased, elevation of the kinase activity was only moderate. These data demonstrate that serine 338 phosphorylation does not necessarily correlate with the extent of kinase activity. Obviously, other pathways are required to convert C-RAF from the inactive to the active state.

**Active Forms of C-RAF Mutants Are Located Preferentially at Membranes—Translocation of cytosolically located RAF kinases to the plasma membrane has been initially proposed to be mediated solely by GTP-loaded Ras proteins via interactions with Ras binding domain (31, 32). We and others provided evidence that RAF kinases *per se* possess high affinities for certain membrane lipids, supporting a new model in which a small fraction of RAF molecules is already associated with the plasma membrane, where Ras-RAF interactions subsequently take place by lateral diffusion in the plane of the membrane (12, 33). To investigate whether the mutations in the N-region influence the translocation of C-RAF kinase to cell membranes, we examined the subcellular localization of C-RAF WT and C-RAF mutants listed in Fig. 1B. In parallel, we determined the serine 338 phosphorylation status and the kinase activity of C-RAF proteins in different cell fractions. To this end COS7 cells were transfected with plasmids expressing Myc-tagged C-RAF wild type and C-RAF substitution mutants listed in Fig. 1B. In this experiment we included C-RAF-Y340D/Y341D (C-RAF-DD) mutant, because this substitution mutant is known to be highly active as well as strongly phosphorylated at serine 338 under nonstimulating conditions, features that we also found with C-RAF-S339G and C-RAF-Q335R mutants. Following serum starvation, subcellular fractions were collected according to the protocol of the ProteoExtract subcellular proteome extraction kit (Calbiochem). The extraction procedure yields four subproteome fractions as follows: cytosolic, membrane, nuclear, and cytoskeletal fractions. C-RAF proteins were absent from the nuclear and cytoskeletal fractions and were exclusively detected in the cytosolic and membrane fractions (see Fig. 6A). However, under nonstimulating conditions more C-RAF protein accumulated in the cytosolic fraction compared with isolated membranes. Because we did not observe significant differences in distribution of C-RAF mutants between cytoplasm and membranes, we next looked at the distribution of their kinase activity. The extraction of cytosolic and membrane fractions yields C-RAF proteins in their native state, including enzyme activity, because control experiments showed that RAF activity was not affected by extraction buffers (data not shown). The extent of C-RAF kinase activity in membrane and cytosolic fractions was determined by use of MEK/ERK-coupled kinase assay. Because the quantities of C-RAF protein differed between fractions, the values of observed kinase activity were normalized for C-RAF content.

As shown in Fig. 6, B and C, most of the kinase activity of the C-RAF-DD mutant was associated with the membrane fraction (57%), in contrast to only 29% of the kinase activity for C-RAF WT. Importantly, C-RAF-Q335Y mutant exhibiting low basal activity behaved similarly to C-RAF WT (Fig. 6, B and C). On the other hand, the degree of membrane-bound RAF activity was increased significantly in the case of C-RAF mutants with elevated basal activity (C-RAF-S339G and C-RAF-Q335R).

We also examined the distribution of C-RAF serine 338 phosphorylation between cytosolic and membrane fractions.
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The quantities of phosphorylated protein were determined by anti-Ser(P)-338 antibody, and the values of observed phosphorylation were normalized for C-RAF content. This examination could only be performed with C-RAF-Q335R and C-RAF-DD mutants, because the phosphorylation degree of C-RAF WT and C-RAF-Q335Y mutants was below the limit of detection. As shown in Fig. 6, D and E, C-RAF phosphorylated on serine 338 was associated preferentially with the membrane fraction, confirming previous findings, suggesting that phosphorylation of serine 338 and tyrosine 341 contributes to Ras-independent membrane localization (25).

These results indicate that mutations in the N-region do not impair translocation of C-RAF protein to the membrane fraction. Moreover, they document again the importance of the nonconserved positions 335 and 339 in C-RAF and show that the C-RAF-S339G and C-RAF-Q335R mutants reveal similar properties, as have been reported for the constitutively active C-RAF-DD, at least with regard to membrane-associated kinase activity.

A Model Derived from the Tertiary Structure of RAF Reveals a Tight Contact between the N-region of A-RAF and Catalytic Domain—To correlate the experimental results presented in this study (see Figs. 2–5) with the steric constellation of the N-region and the catalytic domain, we searched for putative contact points between these two moieties by molecular modeling. For this purpose we took advantage of the recently published three-dimensional structure of B-RAF catalytic domain (34). The validity of this approach is supported by the high degree of homology of the catalytic domain between A-, B-, and C-RAF. The C-terminal part of RAF (CR3) has 96% identity among human isoforms and 95% identity between human and mouse enzymes. In contrast, the similarity among RAF isoforms in their N-terminal portions is only 60%. As shown in Fig. 7A, several contact points could be identified between the catalytic domain and the N-regions in A- and C-RAF. However, there are differences in the interaction patterns between these two RAF isoforms. In both A- and C-RAF the highly conserved aspartate of the N-region
(Asp-337 in C-RAF and Asp-298 in A-RAF) interacts with the spatially adjacent lysine (Lys-399 in C-RAF and Lys-360 in A-RAF) from the αC helix of the catalytic domain. Furthermore, in both A- and C-RAF the oxygen of the backbone of the N-region interacts with the side chain of the conserved arginine (Arg-398 in C-RAF and Arg-359 in A-RAF) from the αC helix. However, in the case of C-RAF the arginine 398 of the catalytic domain makes an additional hydrogen bond to the side chain of the serine 339 of the N-region (Fig. 7A, upper panel). Importantly, substitution of C-RAF serine 339 by glycine (as is the case in the C-RAF-S339G mutant) abrogates this C-RAF-specific hydrogen bond between the N-region and the catalytic domain (Fig. 7C, right panel), whereas introduction of serine instead of glycine 300 (as is the case in the A-RAF-G300S) generates the analogous interaction in A-RAF (Fig. 7B, right panel). These observations together with the experimental results of this study suggest that the presence of a hydrogen bond between the side chain of the serine in position +1 relative to the conserved serine and arginine of the catalytic domain may be inhibitory for RAF kinase activity. Disruption of this close contact between N-region and catalytic domain results in highly elevated basal activity, as has been demonstrated for C-RAF-S339G mutant (Figs. 4 and 5), whereas introduction of this additional hydrogen bond in A-RAF results in completely inhibited kinase, as has been shown for A-RAF-G300S (Fig. 3A).

Furthermore, the modeling data presented in Fig. 7 revealed that the presence of tyrosine at position 296 confers very specific properties onto A-RAF among the RAF kinase family. As illustrated in Fig. 7, A (upper panel) and B, the aromatic group of tyrosine 296 interacts with the backbone of lysine 423 from the αE helix of the catalytic domain. Moreover, this interaction is further strengthened by the hydrogen bond between the side chain of the lysine 423 from the αE helix and the backbone oxygen from the N-region, conferring a tighter contact between N-region and catalytic domain in A-RAF compared with C-RAF, in which these two interactions are missing, because of a glutamine residue (Gln-335) in the analogous position (Fig. 7, A, lower panel, and C). Obviously, tyrosine in this position favors a spatial orientation of the N-region segment, which enables a more close fitting contact with catalytic domain, whereas glutamine in this position partially weakens this interaction. According to this observation, we suggest that the tyrosine 296, which is unique for A-RAF kinase, contributes considerably to the low activating potency of A-RAF, caused not only by significantly lower probability of the serine 299 phosphorylation but also by sterical reasons.

Finally, Connolly surface presentations (Fig. 7A, right panels) revealed not only the conformational differences between A- and C-RAF but also differences in the pattern of the electrostatic potential. The area formed by interaction between N-region and catalytic domain in A-RAF reveals significantly higher electrostatic potential compared with the same area in C-RAF, suggesting that this A-RAF specific feature may also contribute to the low activating potency of this isoform.

Taken together, our results, which have been obtained using a reconstruction model, suggest that the tight binding between the N-region and the catalytic domain acts inhibitory with regard to the kinase activity of RAF proteins, whereas release of this interaction favors the active form of kinase.

**DISCUSSION**

Regulation of RAF activity is a highly complex process, involving association with membrane lipids, interaction with regulatory and scaffold proteins, and numerous phosphorylation events (8). Although the basic activation steps are similar for all three RAF isoforms, there are several subtle differences in the regulation of their kinase activity. These isoform-specific regulation varieties result in the enormous differences between the RAF isoforms with respect to basal activity and activities caused by external stimulation. For example, the maximal activation of B-RAF merely requires signals that generate Ras-GTP, whereas activation of C-RAF and A-RAF requires Ras-GTP together with signals that lead to their phosphorylation on tyrosines in the N-region (26). The N-region, which contains several conserved phosphorylation sites, plays an important role in the course of RAF activation process. Phosphorylation of Ser-338 and Tyr-341 in the N-region initiated by activated Ras and Src family kinases, respectively, is required for full C-RAF activity, whereas Ser-446 (the B-RAF equivalent of Ser-338 in C-RAF) is constitutively phosphorylated, and this phosphorylation, together with the aspartic acids at 448/449 (equivalent to Tyr-340/Tyr-341 of C-RAF), contributes to the high basal activity of B-RAF (24, 27). Comparably little is known about the phosphorylation in the N-region of A-RAF. Substitution of tyrosines 301/302 in A-RAF (equivalent to Tyr-340/Tyr-341 of C-RAF) with aspartic acids generated a protein, which had an elevated basal level of activity, suggesting that these residues play a similar role in the activating process as in C-RAF (26). However, no data are available about the phosphorylation of...
the regulatory serine 299 (equivalent of Ser-338 in C-RAF), and which role this serine plays in the activation process of A-RAF kinase. Furthermore, the contribution of serine 339 in C-RAF and the analogous serine 447 in B-RAF with regard to regulation of RAF kinase activity is controversial. Serine 339 has been reported to be phosphorylated in C-RAF. However, phosphorylation of this site is not required for kinase activation, because the alanine substitution at Ser-339 did not affect C-RAF kinase activity (27).

Although the sequence of N-region is highly conserved between all three RAF isoforms, there are several amino acid variations (Fig. 1A). The relevance of aspartate residues in the B-RAF N-region at positions that are occupied by tyrosines in A- and C-RAF for B-RAF kinase activity is already well known. In contrast, the role of the glycine in the N-region of A-RAF in position 300, which is occupied by serine in B- and C-RAF, in activation of A-RAF has not been investigated so far. Similarly, the possible contribution of the amino acid variations at the position −3 to the highly conserved serine (Ser-299 in A-RAF, Ser-338 in C-RAF, and Ser-446 in B-RAF) for its phosphorylation and RAF activation has not been studied. Therefore, in this study we focused on the differences in the structure of the N-region and addressed the question whether these nonconserved residues in positions −3 and +1 relative to the highly conserved serine participate in RAF activation. Here we report that nonconserved residues that have not been considered as regulatory sites determine to a considerable extent the RAF activation process. In particular, the residues $X_1$ and $X_2$ in the core structure of the N-region GX,RDSX$_3$YY(DD)WE strongly influence the magnitude and dynamics of RAF activation. We found, contrary to expectation, that glycine in position 300 affected the activation of A-RAF in a stimulatory manner. This finding was corroborated...
ated by the observation that substitution of serine 339 by glycine in C-RAF led to a constitutively active kinase (Fig. 4 and Fig. 5, A–C), thus confirming the assumption that glycine in position +1 relative to Ser-338 facilitates RAF activation. Our results demonstrate further that the substitution of glycine 300 with serine in A-RAF is inhibitory for A-RAF kinase activity (Fig. 3, A and B). The importance of the glycine at this position is quite surprising, because analysis of A-RAF protein sequences from different species has shown that only mammalian A-RAF contains glycine at position 300, whereas in frogs and fishes A-RAF contains a serine residue at this position, similar to the sequences of C- and B-RAF in this region. Because the introduction of glycine instead of serine in position X_3 only impairs the recognition sequence of the anti-Ser(P)-338 antibody, we were not able using this antibody to examine whether glycine at this position enhances RAF kinase activity by facilitating the phosphorylation of the conserved serine. On the other hand, substitution of glycine with serine in A-RAF did not impair the phosphorylation of the neighboring tyrosines 301/302 upon activation with Lck (Fig. 3D and supplemental Fig. S2), suggesting that amino acid X_3 influences only the Ras-GTP-dependent RAF activation pathway but not the Src-dependent RAF kinase activation.

Furthermore, we also showed in this study that amino acid in the position −3 relative to conserved serine influences the phosphorylation efficiency of this serine residue. Whereas glutamine in this position leads to moderate phosphorylation and tyrosine almost completely abrogates phosphorylation of conserved serine, introduction of arginine in the same position increases enormously the phosphorylation efficiency and consequently the activity of RAF kinases (Fig. 3, A and C, Fig. 4, and Fig. 5, B and D).

These results raise the following question: which of the protein kinases that phosphorylate the conserved serine in the N-region of RAF is dependent on amino acid X_3? p21-activated protein kinase PAK3 has been reported to be phosphorylated by C-RAF on serine Ser-338 in vitro and in vivo (35). Moreover, Ser-338 phosphorylation by PAK was found to be very sensitive to loss of N-terminal arginines resulting in decreased phosphorylation (36). However, it was not examined whether Ser-338 phosphorylation by PAK is also sensitive to alterations in amino acid side chains in position −3 to this serine. Furthermore, Chiloeches et al. (37) have shown that PAK3 is not activated under conditions where Ser-338 is phosphorylated, but when PAK3 is strongly activated, either by co-expression with Cdc42–12V or by mutations, which render it independent from Cdc42, it did stimulate Ser-338 phosphorylation. The role of this kinase as a physiological mediator of Ser-338 phosphorylation in growth factor-stimulated cells therefore remains highly controversial. Recently, serine-threonine kinase CK2 was identified as a RAF family N-region kinase. Using in vitro kinase assay, it was shown that CK2 efficiently phosphorylates B-RAF on serine 446. On the other hand, CK2 can only phosphorylate C-RAF that has been co-expressed with v-Src or contained an aspartic acid residue at the position 341, indicating that the conserved serine is a target of CK2 only when a negatively charged residue is additionally present at the +3 position (38). It is unlikely that the amino acid variations at the position −3 would impair phosphorylation of conserved serine in the N-region of RAF by CK2, because, according to the consensus target sequence for this kinase ((S/T)XX(E/D/pY/pS/pPT)), the negatively charged amino acids C-terminal rather than the amino acid constellation N-terminal to the phosphorylation site are crucial for substrate recognition (39). Using NetPhosK 1.0 server (40), which produces neural network predictions of kinase-specific eukaryotic protein phosphorylation sites, we looked for the further putative serine-threonine kinases that could be proposed to phosphorylate RAF proteins on the conserved serine within the N-region. Currently, NetPhosK 1.0 covers the following kinases: PKA, protein kinase C, protein kinase G, CKII, Cdc2, CaM-II, ATM, DNA PK, Cdk5, p38 MAPK, GSK3, CKI, protein kinase B, RSK, INSR, EGF receptor, and Src. Besides CK2, PKA and p90 ribosomal Ser-6 kinase (RSK) are also predicted to be potential kinases for phosphorylation on conserved serine within the RAF N-region (Table 1). B-RAF WT is predicted to be efficiently phosphorylated by both PKA and RSK, whereas C-RAF WT is phosphorylated by PKA only. A-RAF WT is not phosphorylated at all. Introduction of arginine instead of glutamine at position 335 in C-RAF results in a protein, which is predicted to be phosphorylated on serine 338 by PKA as well as by RSK, which presumably is the basis of the increased kinase activity of this mutant relative to C-RAF WT (Table 1). Furthermore, according to NetPhosK 1.0 analysis A-RAF-Y296R mutant is predicted to be highly phosphorylated by PKA kinase in contrast to A-RAF.

**TABLE 1**

| RAF isoforms and mutants | N-region sequence | Predicted kinases | Phosphorylation score |
|-------------------------|------------------|-------------------|---------------------|
| B-RAF WT | LGRRDSSDD | PKA | 72 |
| C-RAF WT | RGRSDSYY | PKA | 63 |
| (Q335R) | RGRDDSYY | PKA | 84 |
| A-RAF WT | LGYRSDDGYY | Nonphosphorylated |
| A-RAF | LGRRDSSYY | PKA | 87 |

| Protein kinase | Consensus sequences |
|----------------|---------------------|
| PKA | R(R/K)(X)(S/T) > (R(X)(S/T)) (33, 44) |
| R(S/T) | (R/R/X)(S/T) > (K/R)(X)(S/T) (45, 46) |

**FIGURE 7.** Reconstruction of spatial orientation of the N-region relative to the catalytic domain in A- and C-RAF utilizing three-dimensional structure of the B-RAF catalytic domain as a template. A, ribbons diagram and Connolly surface of the reconstructed three-dimensional structure of A-RAF (upper panel) and C-RAF (lower panel). The positions of Tyr-296, Arg-297, Asp-298, Ser-299, Gly-300, Tyr-301, and Tyr-302 of the N-region and the positions of Lys-423, Arg-359, Lys-360, and Arg-362 of the catalytic domain in A-RAF as well as the positions of the corresponding amino acids in C-RAF are indicated. ATP-binding site and active site are in purple; αC helix is in light blue; αE helix is in green. The area formed by interaction between N-region and catalytic domain within the Connolly surface presentation is indicated by black rectangle. B, alterations of hydrogen bonds between the N-region and the catalytic domain in A-RAF wild type and A-RAF-G300S mutant (close-up view). C, alterations of hydrogen bonds between the N-region and the catalytic domain in C-RAF wild type and C-RAF-S339G mutant (close-up view).
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WT, which is not phosphorylated at all. These predictions are in accordance with our experimental data and may present a potential explanation, i.e. how the amino acid at the position −3 to the conserved serine within the N-region influences its phosphorylation in the A- and C-RAF substitution mutants examined in this study.

Taking advantage of the recently published three-dimensional structure of the B-RAF catalytic domain (34), we provide a potential answer to the following question: how to explain the effects of substitutions in positions X1 and X2 on RAF kinase activity on the molecular basis considering the three-dimensional structure of RAF kinases? Although the exact three-dimensional structure of the complete RAF protein is still unknown, it has been generally accepted that the N-terminal regulatory part in A- and C-RAF associates with the catalytic domain in the basal state forming a closed conformation of the protein (5). On the other hand, it is very probable that contact points between N-region and both the regulatory moiety (including CR1 and CR2) and the catalytic domain exist, because N-region is positioned exactly between these two domains. Using molecular modeling, we reconstructed the possible spatial orientation of the N-region in A- and C-RAF relative to the catalytic domain utilizing three-dimensional structure of the B-RAF catalytic domain as a template.

According to the model illustrated in Fig. 7A, we identified contact points between N-region and the αC and αE helices of the catalytic domain. Taking into account our experimental results, this contact is proposed to regulate RAF kinase activity allowing tight interaction between the N-region and the catalytic domain and inhibiting RAF activation, whereas weakening of this binding enhances RAF kinase activity. Indeed, disruption of the hydrogen bond between serine 339 of the N-region and arginine 398 of the catalytic domain of C-RAF by serine to glycine substitution led to constitutively active C-RAF (C-RAF-S339G and C-RAF-Q335Y/S339G mutants, see also Figs. 4, 5, and 7C), whereas induction of this interaction by reverse amino acid exchange in A-RAF inhibited kinase activity (A-RAF-G300S and A-RAF-Y296R/G300S mutants, see also Figs. 3, A and B, and 7B). Furthermore, it should be considered that glycine is the only amino acid without a side chain. Introduction of this amino acid into a protein sequence renders its structure more flexible. In our case the introduction of glycine into the N-region between the conserved serine and two tyrosines has a dual effect. First, as already mentioned, it disrupts a hydrogen bond to the αC helix of the catalytic domain. Second, it also increases flexibility to the adjacent segment and by this means further weakens hydrogen binding to the catalytic domain. This assumption has been confirmed by the observation that anti-Ser(P)-338 antibody, which recognizes Ser(P)-338 in C-RAF and Ser(P)-446 in B-RAF, cannot recognize Ser(P)-299 in A-RAF, probably because of the difference in the structure and conformation of these epitopes.

Furthermore, the three-dimensional model presented in Fig. 7 together with our experimental results reveal that amino acids at position 296 in A-RAF may also have a dual effect on RAF kinase activity. A tyrosine residue at this position decreases the kinase activity because of inhibition of phosphorylation on serine 299 and, additionally, because of its interaction with the αE helix of the catalytic domain. On the other hand, arginine at the same position facilitates serine phosphorylation and disrupts the contact to the αE helix of catalytic domain; both effects reinforce the RAF kinase activity.

In contrast, according to the reconstruction model (Fig. 7), the phosphoserine at position 338 in C-RAF (299 in A-RAF) and phosphotyrosines at positions 340/341 in C-RAF (301/302 in A-RAF) would rather interact with the regulatory domain than with the catalytic domain, because the side chains of these residues are orientated out of the plane in which the N-region interacts with the catalytic domain. Interaction of these phosphorylated residues with the regulatory part of the protein may pull away the N-region segment and contribute to the disruption of the contact between N-region and catalytic domain, resulting in elevated kinase activity.

The consequences of the abolishment of the binding between the N-region and the catalytic domain of RAF may be the reorganization in the complex formation between 14-3-3 proteins and RAF and subsequently preferential formation of heterodimers between C- and B-RAF (14–16). Another possible consequence may involve Ras-independent recruitment of RAF to membranes. The region of the RAF catalytic domain, which in our reconstruction model makes contacts to the N-region (Fig. 7), is part of the sequence, which has been described as the phosphatidic acid-binding region (PABR) of RAF proteins. The PABR is a highly conserved 36-amino acid region, which has been supposed to associate with phosphatidic acid. It contains a cluster of basic residues (Arg-398, Lys-399, and Arg-401 in C-RAF) as well as a short segment of hydrophobic residues (405–408) (41). Mutation of one of these residues to alanine (R398A) was sufficient to reduce the ability of RAF proteins to associate with phosphatidic acid. However, this observation is limited to insulin-dependent ERK activation localized on endosomal vesicles (33). Interestingly, in our reconstruction model arginine 398 exactly matches the residue, which interacts with the serine 339 of the N-region segment in C-RAF. Considering this observation, it may be possible that disruption of the contact between the N-region and the PABR of the catalytic domain may release this segment for the binding to membrane lipids. This hypothesis would be in accordance with our fractionation data that show the active and the serine 338-phosphorylated C-RAF forms located preferentially at the membranes (Fig. 6). Nevertheless, more questions remain open, because the three-dimensional structure of the N-terminal regulatory part is still not known. Therefore, at present time we can only speculate whether additional contact points between N-region and regulatory domains of RAF kinases exist.

Results presented in this contribution raise the old questions: why do most vertebrates have three RAF genes and what is the gain of function of this situation relative to insects and nematodes that make do with just one RAF gene? One possibility is that having three RAF enzymes with widely differing basal and inducible activities (42) could significantly improve the fine-tuning of the mitogenic cascade. This principle is reminiscent of the EGF receptor family, which has four members, which are strikingly different in their regulation. Because of its similarities with D-RAF and Lin-45, B-RAF is most closely related to soli-
The transition of B-RAF to C- and A-RAF probably required an attenuation of the extraordinary high basal activity of B-RAF. This has been primarily achieved by replacement of the two aspartic acids of N-region at the positions 448 and 449 (Asp-448 and Asp-449) by tyrosines that could now be regulated. Our data presented here indicate that the replacement of arginine 443 of B-RAF by glutamine, as is the case in C-RAF (Gln-335), led to further reduction of basal and inducible activity with specific mutations in the N-region resulted in the fine regulation of the basal and inducible kinase activities of RAF. It appears that in the course of evolution to introduce glycine instead of serine in the consecutive shortening of the N-terminal part together with specific mutations in the N-region resulted in the fine regulation of the basal and inducible kinase activities of RAF isoforms.

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