In mammals the RAF family of serine/threonine kinases consists of three members, A-, B-, and C-RAF. Activation of RAF kinases involves a complex series of phosphorylations. While the most prominent phosphorylation sites of B- and C-RAF are well characterized, little is known about regulatory phosphorylation of A-RAF. Using mass spectrometry we identified here a number of novel in vivo phosphorylation sites in A-RAF. In particular, we found that S432 participates in MEK binding and is indispensable for A-RAF signaling. On the other hand, phosphorylation within the activation segment does not contribute to EGF-mediated activation. Furthermore, we show that the potential 14-3-3 binding domains in A-RAF are phosphorylated independently of its activation status. Of importance, we identified a novel regulatory domain in A-RAF (referred to as IH-segment) positioned between amino acids 248 and 267 that contains seven putative phosphorylation sites. Three of these sites, serines 257, 262 and 264, regulate A-RAF activation in a stimulatory manner. The spatial model of the A-RAF fragment comprising residues between S246 and E277 revealed switch of charge at the molecular surface of IH-region upon phosphorylation, suggesting a mechanism in which the high accumulation of negative charges may lead to an electrostatic destabilization of protein-membrane interaction resulting in depletion of A-RAF from the plasma membrane. Together, we provide here for the first time a detailed analysis of in vivo A-RAF phosphorylation status and demonstrate that regulation of A-RAF by phosphorylation exhibits unique features compared to B- and C-RAF.

RAF proteins, originally discovered as the oncogenic product of mouse sarcoma virus 3611 (1), belong to the family of serine/threonine-specific protein kinases that control several normal as well as pathologic processes including cell proliferation, transformation, differentiation and apoptosis (2-4). RAF kinases serve as the key modules for the classical mitogenic signal transduction cascade, functioning to connect receptor tyrosine kinases via MEK and ERK with nuclear transcription factors. Members of RAF protein family are present in several multicellular organisms (5-8). Unlike lower eukaryotes, which express only one RAF gene, vertebrates inherit three members of the RAF family of proto-oncogenes, A-RAF, B-RAF, and C-RAF (8-11).

All RAF isoforms share three conserved regions (CR1, CR2, and CR3) and can be divided in two functional parts, the amino-terminal regulatory domain and the carboxy-terminal kinase domain (9). The regulatory part includes CR1 and CR2 that in the case of C-RAF span residues 51–194 (CR1) and 254–269 (CR2). The CR1 consists of Ras-binding domain (RBD) followed by cysteine-rich domain (CRD), whereas CR2 is rich in serines and threonines. CR3 represents the kinase domain extending in C-RAF from residues 347 to 613 (see also Fig. 1A). Despite intensive investigations, the molecular mechanism by which RAF activity is regulated is not completely elucidated. Although direct experimental support is missing, it has been generally accepted that the N-terminal regulatory part of C-RAF interacts in non-stimulated cells...
with catalytic domain, promoting a closed conformation of the kinase. Association with 14-3-3 proteins may further stabilize this inactive conformation (12). A current model suggests that RAF association with plasma membrane lipids or lipid rafts represents the prerequisite step in the RAF activation (13,14). Upon stimulation of cell surface receptors association with the activated small G-protein Ras (Ras-GTP) occurs in the plane of the inner leaflet of plasma membrane. Binding to activated Ras initiates further activation steps such as phosphorylation events, differential association with 14-3-3 proteins and finally dimer formation with B-RAF (15-19).

Phosphorylation of RAF kinases is of particular importance in RAF regulation, as it plays a crucial role not only in the activation process but is also a prerequisite for docking of several interacting partners such as 14-3-3 proteins and MEK. In C- and B-RAF both stimulatory and inhibitory sites have been reported. In C-RAF the most prominent basal phosphorylation sites have been identified as serines 43, 259, and 621. These sites have been shown to be phosphorylated in growth factor stimulated as well as in resting cells (20). Phosphorylation of serines in positions 259 and 621 is necessary for association with 14-3-3 proteins (21). Phosphorylation of the internal 14-3-3 binding site serine 259 has been reported to suppress C-RAF kinase activity (20,22-24), whereas regulation of C-RAF kinase activity by serine 621 phosphorylation is controversially discussed (25,26). Furthermore, Dumaz et al. (27) reported that in addition to serine 259 cAMP dependent phosphorylation of serines 43 and 233 by protein kinase A results in negative regulation of C-RAF function. Recently, serine 471 in C-RAF and corresponding residue in B-RAF (S578) were identified as in vivo phosphorylation sites that are critical for RAF activity and serve as a docking site for MEK (28).

The highly conserved motif DFGLATVKSR, which is a part of the activation segment, is also subject to regulation by phosphorylation of residues T491 and S494 in C-RAF. They are homologous to T452 and T455 of A-RAF, as well as to T599 and S602 of B-RAF. This segment lies within the putative activation loop of the kinase domain and is likely to be necessary for kinase activity of B- and C-RAF (29,30). The importance of these conserved residues for A-RAF activation remains to be investigated.

Treatment of cells with growth factors induces phosphorylation of C-RAF at multiple sites located within the regulatory part of the protein. These are threonines 268/269, serines 289/296/301, serines 338/339, and tyrosines 340/341. Threonine 268 within the CR2 domain in C-RAF and the homologous residue threonine 372 in B-RAF represent an autophosphorylation site (20,31), whereas threonine 269 of C-RAF has been proposed to serve as a target for phosphorylation by KSR (32). Recently, we and other groups (33-35) detected novel phosphorylation sites at three SP motifs of C-RAF (serines 289, 296, and 301). They are located within the variable sequence stretch, which connects CR2 with kinase domain. Although the interpretation of the results is still controversial the published data document that these sites regulate C-RAF by direct ERK-mediated feedback phosphorylation (34,35).

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 (S299 in A-RAF, S338 in C-RAF, and S446 in B-RAF) and two conserved tyrosines (Y301/Y302 in A-RAF, and Y340/Y341 in C-RAF), which are substituted to aspartates in B-RAF. Phosphorylation of these sites positively regulates kinase activity of RAF kinases (36-39). Corroborating the complex regulation of RAF kinase activity by the N-region we recently showed that beside the regulatory function of serine 338 of C-RAF (and corresponding residues in A- and B-RAF) the non-conserved amino acids in positions -3 and +1 relative to S338 play also an important role in the course of RAF activation (39).

Regarding regulation of B-RAF activity by phosphorylation there are similarities, but also essential differences compared to C-RAF. Phosphorylation of serines 365 and 729 that are equivalent to serines 259 and 621 of C-RAF has been shown to mediate 14-3-3 binding to B-RAF and regulate its activity in the similar way as reported for C-RAF (16,40). On the other hand,
the N-region mediated regulation is quite different in B- and C-RAF because the tyrosine residues of C-RAF (Y340 and Y341) are occupied by aspartic acids in B-RAFT (D448 and D449). This leads to constitutive phosphorylation of the serine 446 in B-RAFT (3). Thus, due to the accumulated negative charge at the N-region the B-RAF kinase exhibits unusual high basal kinase activity (13,38).

With some exceptions little is known about the role of phosphorylation in the activation process of A-RAF. We showed recently that the presence of serine 299 and within the N-region is necessary for A-RAF activation (39). In order to address how phosphorylation events apart from N-region regulate the activation cycle of A-RAF, we performed here a systematic analysis of the potential phosphorylation sites in A-RAF protein by use of mass spectrometry. The physiological role and the function of the novel phosphorylation sites derived from the mass spectrometry data have been investigated subsequently by amino acid exchange at the relevant positions. In particular, we report here that, similar to B- and C-RAF, the potential 14-3-3 binding domains in A-RAF are phosphorylated at the serines 214 and 582 independent of its activation status. Furthermore, we found that serine 432 within the potential MEK binding domain is essential for A-RAF signaling, whereas the importance of phosphorylation within the activation segment (threonines 452 and 455) for A-RAF activity is restricted to the Ras/Lck-mediated stimulation. In addition, we identified seven novel phosphorylation sites within a triptic peptide corresponding to A-RAF 248-267. We designate this regulatory domain as IH-segment (Isoform-specific Hinge segment). Three of the phosphorylation sites within this segment (serines 257, 262, and 264) are strongly involved in the positive regulation of A-RAF activity. The spatial model of the A-RAF fragment comprising residues between serine 246 and glutamine 277 revealed switch of charge at the molecular surface of IH-region upon phosphorylation, suggesting a mechanism in which the high accumulation of negative charges may lead to an electrostatic destabilization of protein-membrane interaction resulting in depletion of A-RAF from the plasma membrane upon prolonged stimulation. Collectively, we present here for the first time a detailed analysis of the A-RAF phosphorylation that together with data recently published by our group (39) illuminate the complex RAF activation process.

**Experimental Procedures**

**Materials** – EGF was from CellSystems and U0126 from Promega. Anti-phospho-ERK, anti-Myc, anti-ERK, anti-actin, and anti-Lck antibodies were from Santa Cruz Biotechnology. Antibody against H-Ras was from Transduction Laboratories. Phosphospecific antibodies directed against Ser(P)-259 and Ser(P)-338 of C-RAF were from Upstate Biotechnology and Cell Signaling, respectively. Anti-Ser(P)-621 of C-RAF antibody was generated in-house (16). Horseradish peroxidase-conjugated polyclonal anti-rabbit and anti-mouse IgG were obtained from Dianova. Anti-M2PK was from Schebo Biotech. Anti-histon 3 antibody was obtained from Apstate (kindly provided by M. Becker). Antibody against vimentin was from Dako (kindly provided by R. Houber).

**DNA construction** – Cloning of C-terminal Myc-tagged human A- and C-RAF was performed as previously described (39). The site-specific mutations of A-RAF were introduced using QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The fidelity of the A-RAF mutants was confirmed by DNA sequencing.

**Cell culture, transfection, and immunoprecipitation** – COS7 cells were grown in DMEM (Invitrogen) containing 10% FCS, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin at 37°C, in humidified air with 5% CO2. cDNA encoding the Myc-tagged A-RAF was transfected either alone or with H-Ras12V and Lck into COS7 cells using jetPEI transfection reagent (Biomol); a total of 8 µg of recombinant DNA per 10 cm Petri dish was used. The transfection was carried out under starvation conditions in DMEM containing 0.03 % FCS for 24 h, followed by lysis with buffer containing 50 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% (v/v) glycerol, 0.1% (v/v) β-mercaptoethanol, 25 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM Na3VO4, 25 mM NaF, 1% Nonidet P-40 (NP-40), and cocktail of standard proteinase inhibitors for 45 min at 4°C. In some experiments...
cells were incubated with 100 ng/ml EGF or 10 µM U0126 for times indicated before lysis. All lysates were clarified by centrifugation at 27,000 x 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% NP-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 as previously described (39). To quantify the Western blot signals (e.g., A-RAF and pA-RAF, ERK and pERK) X-ray films (FujiFilm) were scanned and optical density was determined by use of ImageJ software.

**Infection of Sf9 insect cells, purification of A-RAF kinase, SDS–PAGE and Western blot analysis** – For the production 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 PBS-buffer and pelleted at 1100 rpm. Lysis and purification of GST-tagged A-RAF proteins were performed as described by Hekman et al. (16). The purity of A-RAF 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 with antibodies specific for A-RAF.

**Subcellular fractionation** – Cell fractions were isolated using the ProteoExtract subcellular proteome extraction kit (Calbiochem). COS7 cells were grown on the 10-cm Petri dishes, transfected with cDNAs encoding Myc-tagged A- and C-RAF proteins in the presence and absence of H-Ras12V and Lck. 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 RAF proteins were detected by anti-Myc antibody. The selectivity of subcellular extraction was documented by immunoblotting against marker proteins (M2PK for cytosolic fraction, histon 3 for nuclear fraction, and vimentin for cytoskeletal fraction).

**Modeling of the three-dimensional structure of the IH-segment in A-RAF** – Structure models for the IH-segment of A-RAF comprising residues S246 to E277 were obtained by de novo modeling using the software QUANTA2006 (Accelrys Inc, San Diego). Briefly, the peptide sequence was built using the Sequence Builder in the ProteinDesign tool of QUANTA2006 with the peptide adopting an extended conformation. The sequence of the phosphopeptide was built in the identical manner using phospho-serines and -threonine for residues S250, T253, S257, S259, S262, S264, and S265. Secondary structure prediction using the software ProteinPredict (41), JPred (42) and AGSSP (http://imtech.res.in/raghava/appsp/) was performed to test whether the IH-segment of A-RAF might adopt a defined secondary structure. However, none of the programs predicted defined secondary structure elements independent whether this or longer stretches covering the region of interest were used in the prediction. Thus, molecular dynamics simulation was used to determine possible differences due to the phosphorylation. First shorter simulations starting with an extended peptide were performed in vacuo using the CHARMM22 force field including electrostatic terms but with a constant dielectric constant ε set to 78 in water. Simulations of 200ps were performed causing the extended peptide to collapse to a more globular structure. These globular structures were put into explicit solvent by immersing the peptide structures in water using a water shell of 10Å around the peptide. After energy minimization using 1000 steps of conjugate gradient minimization, molecular dynamics simulations of 500ps length were performed employing the CHARMM22 force field. Several runs were conducted to determine the probabilities of defined structure elements obtained in these simulations. No defined structures could be obtained by these simulations, which is consistent with the secondary structure predictions. Calculation of the electrostatic potential maps of the phosphorylated and non-phosphorylated peptide was carried out using the software ABPS (43). The charge force field of AMBER7.0 was used. Charges for phospho-serine and -threonine were obtained from Homeyer et al. (44). Potential maps were calculated using an ionic strength of 0 mM,
however using physiological salt concentrations of 150 mM did not alter the maps other than scaling the potentials to the higher salt. Charge distribution was identical at different salt concentrations.

Mass spectrometry measurements – Purified GST-tagged A-RAF samples (about 10 pmol of each) were applied to SDS-PAGE. Proteins were visualized by Coomassie Blue staining applying the method of Neuhoff et al. (45). In-gel reduction, acetylation, trypsic and/or GluC digestion were done according to Wilm et al. (46). After elution of the peptides, solutions were desalted using Millipore C18 ZipTip according to the manufacturer’s instructions. ESI-MS was performed on a Bruker APEX II FT-ICR mass spectrometer (Bruker Daltonic GmbH Bremen) equipped with an Apollo-Nano-ESI ion source in positive ion mode. To determine the exact positions of phosphates within the peptides the nano-LC-MS/MS analysis was carried out as follows: A-RAF samples were separated by SDS-PAGE, protein bands were excised, washed, digested and generated peptides were extracted as described in (47). Nano-LC-MS/MS analyses and transformation of raw data into MGF format were conducted as described in (48) on a Qstar Elite (Applied Biosystems, Darmstadt, Germany) and an LTQ XL (Thermo Scientific, Dreieich, Germany) mass analyzer in multistage mode, respectively. Generated peak lists were searched using Mascot 2.2 against a SGD protein database with the concatenated A-RAF protein sequence (a total of 6,318 sequences) using the following criteria: trypsin with a maximum of 2 missed cleavage sites, carboxidimethylation of cysteine residues as fixed and phosphorylation of serine, threonine, and tyrosine residues as variable modifications. For the Qstar Elite, mass tolerances were set to 0.1 Da for MS and MS/MS, whereas for the LTQ XL, mass tolerances were set to 0.3 Da for MS as well as 2.0 for MS/MS. Additionally, the $^{13}$C option was set to 1 to account for the potentially inaccurate assignment of monoisotopic peaks. All phosphopeptide MS/MS spectra were validated manually as described in (48). Peptides represented in Table 1 were identified reproducibly, whereas a number of further potential phosphopeptides generated MS/MS spectra of inferior quality and therefore were omitted.

RESULTS

MS analysis of A-RAF phosphorylation – Recently we performed a quantitative analysis of C-RAF phosphorylation by use of mass spectrometry that resulted in identification of several new regulatory phosphorylation sites (33). To obtain a comprehensive map of potential phosphorylation sites in A-RAF we used again mass spectrometry. For that purpose, we analyzed A-RAF proteins expressed in the presence and absence of Ras12V and Lck. After trypsin and/or GluC digestion of purified protein and desalting procedures the selective detection of the phosphopeptides has been performed by ESI-MS and nano-LC-MS/MS techniques. Three independent measurements provided 84 % coverage of the entire protein sequence. The combined results obtained for A-RAF phosphorylation are summarized in Table S1 and Fig. 1B revealing a number of novel phosphorylation sites in A-RAF. To compare the mass spectrometry results obtained for A-RAF with the known phosphorylation sites in B- and C-RAF we aligned the amino acid sequences of RAF isoforms as shown in Fig. 1B. Most of the phosphorylated peptides obtained by mass spectrometry are localized within the kinase domain of A-RAF (Fig. 1B and Table 1S). The peptides corresponding to A-RAF 432–444 and 445–456 could be ascribed to the MEK binding domain and activation segment, respectively, due to homology with B- and C-RAF. Furthermore, we detected a number of putative phosphorylation sites within the A-RAF kinase domain, whose function has not previously been elucidated for any RAF kinase. A-RAF peptide 315–326 carrying three phosphate residues at T318, S320, and T323 and peptide 401–423 carrying two phosphates at T413 and Y419 cover the sequences that are highly conserved in all three RAF isoforms. Because the number of phosphates within these two peptides corresponds to the number of phosphorylation possibilities, we propose that all these positions represent putative phosphorylation sites of RAF proteins. Similarly, the number of the detected phosphates in the
partially conserved peptides 340-354, 534-544, and 545-554 correlates with the available phosphorylation sites, suggesting that S341, T344, S535, S536, and S547 may fulfill a regulatory role in A-RAF function (Fig. 1B and Table S1). In contrast, the exact position of the phosphate within the peptide 525–533 has not been specified despite the fragmentation analysis. Furthermore, we detected phosphorylation of three peptides at the very C-terminus of A-RAF protein. One of them (580–588) carrying one or two phosphates corresponds to the highly conserved fragment that has been reported to function as the 14-3-3 binding domain in B- and C-RAF. The second one (589–606) carrying two phosphates cover the sequence that is non-conserved and contains two A-RAF specific phosphorylation sites, T589 and S600. The third peptide (584-603) containing three phosphate residues covers part of the 14-3-3 binding domain (pS585) and the very C-terminal sequence (pS589 and pS600). In addition, we observed phosphorylation at four peptides partially localized within the CR1 domain that contain three putative phosphorylation sites S15, T20, and Y24 (Fig. 1B and Table S1). Whereas S15 is conserved through all three RAF isoforms, T20 is present only in A- and C-RAF, and Y24 appears to be A-RAF specific site. The most surprising finding was the identification of several peptides covering the segment between residues 248–288 located within the non-conserved region between CR2 and CR3 (Fig. 1B and Table S1). This sequence stretch bears up to ten putative phosphorylation sites. Two of these phosphorylation sites serines 257 and 262 are homologous to the serines 296 and 301 of C-RAF that were identified as feed-back regulatory sites (33-35). The remaining phosphorylation sites are A-RAF specific. By use of nano-LC-MS/MS we determined the exact positions of the most novel A-RAF phosphorylation sites described above (see Table 1 and Fig. S2).

To investigate the functional role of the phosphorylation sites detected by MS technique, particularly with respect to regulation of A-RAF activation, we cloned A-RAF proteins, in which serine/threonine residues were substituted by alanine at positions that appear to be of importance for the activation process.

The putative 14-3-3 binding domains in A-RAF are phosphorylated in positions serine 214 and 582 and regulate A-RAF kinase activity – 14-3-3 proteins have been found to support RAF activation (49). On the other hand, it has also been reported that 14-3-3 are not essential for RAF function (50). 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. However, while 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 significantly from the corresponding 14-3-3 binding motifs in A- and C-RAF (RSTpSTP) (see also Fig. 1B).

Association of 14-3-3 proteins with B- and C-RAF has been documented (13,16,40). In contrast, although A-RAF contains the typical 14-3-3 binding domains, little is known about the binding and regulation of A-RAF kinase activity by 14-3-3 proteins. Therefore, we analyzed in this contribution the phosphorylation status of the putative 14-3-3 binding sites in A-RAF. We detected the tryptic peptide SASEPSLHR that contained either one or two phosphate residues (Fig. 1B and Table S1). This peptide corresponds to the A-RAF 580–588 segment and comprises the C-terminal 14-3-3 binding domain. Fragmentation analysis revealed phosphorylations at the serines 582 and 585 (see Table 1 and Fig. S2). In addition, the phosphospecific antibody 6B4 directed against the homologous C-terminal 14-3-3 binding site of C-RAF identified A-RAF wild type, but not the A-RAF-S582A mutant (Fig. 2A). Regarding the internal 14-3-3 binding domain of A-RAF surrounding serine 214 we did not detect a phosphopeptide corresponding to this region. Nevertheless, using phosphospecific antibody directed against internal 14-3-3 binding domain of C-RAF surrounding the S259, that reveals high homology to the internal 14-3-3 binding domain of A-RAF, we show here that A-RAF is phosphorylated at S214. Both, non-activated A-RAF and A-RAF co-expressed with Ras12V and Lck were detectable by use of anti-pS259 antibody (Fig. 2A). Replacement of S214 by alanine completely abolished the phosphate detection by anti-pS259 antibody.

The presence of the serines 621 and 729 in C- and B-RAF respectively has been shown to be necessary for the effective activation of these kinases (16,40). Therefore, we asked, whether phosphorylation of the corresponding serine 582 in
A-RAF exhibits similar effects. To answer this question we examined here the extent of kinase activity of A-RAF WT and A-RAF mutants (A-RAF-S214A and A-RAF-S582A) in the basal state and upon co-expression with Ras12V and Lck. Results shown in Fig. 2A and B document that the presence of the intact C-terminal 14-3-3 binding domain was partially required for effective kinase stimulation by Ras12V and Lck, because the degree of Ras/Lck induced activation of A-RAF-S582A was lower than that of A-RAF WT (Fig. 2). Moreover, the basal activity of A-RAF-S582A was completely abolished upon substitution of serine 582 by alanine. Regarding the second (internal) 14-3-3 binding site it has been reported that substitution of serine 365 by alanine in B-RAF led to a strong increase of kinase activity indicating that the phosphorylation of this site acts highly inhibitory (40). Similar observations have been also obtained for C-RAF; however, the stimulatory effects were lower compared to B-RAF (51). In the case of A-RAF we found that both the basal and the inducible kinase activity of the A-RAF-S214A mutant were considerably elevated compared to the wild type resulting in an active form of A-RAF (Fig. 2). These results suggest that A-RAF activation is strongly influenced by both 14-3-3 binding domain.

Serine 432 is critical for A-RAF kinase activity – As presented in Fig. 1B and Table S1 we detected among others a tryptic peptide with a molecular weight of 1551 Daltons carrying one phosphate. This peptide has been ascribed to the highly conserved region of RAF kinases located between residues 432 and 444 in A-RAF having the following amino acid sequence: SNNIFLHEGLTK. Zhu et al. (28) showed previously that the corresponding sites in C- and B-RAF (S471 and S578, respectively) located within this conserved region are critical for kinase activity and for interaction with MEK. The A-RAF fragment 432–444 possesses two putative phosphorylation sites S432 and T442. However, even the analysis by nano-LC-MS/MS did not provide information, which of these sites is being phosphorylated. To specify the functional significance of these novel phosphorylation sites in A-RAF we examined the effects of mutations at these sites on A-RAF stimulation. To this end, we prepared both A-RAF point mutants, where either S432 or T442 was substituted by alanine (A-RAF-S432A and A-RAF-T442A). To study the kinase activity we transfected A-RAF WT and the A-RAF mutants into COS7 cells. Following EGF stimulation of the cells and immunoprecipitation of A-RAF proteins, kinase activities of the A-RAF mutants were compared with that of A-RAF WT. In order to achieve maximal activation, A-RAF WT and mutants were alternatively stimulated by co-expression with Ras12V and Lck. As demonstrated in Fig. 3A and B in the case of EGF stimulation the exchange of S432 by alanine resulted in a complete abolishment of A-RAF activity, whereas A-RAF-T442A variant exhibited only a moderate reduction of kinase activity compared to A-RAF WT. These data indicate that the A-RAF residue S432, in contrast to T442 is critical for EGF-mediated A-RAF signaling, similar to the findings of Zhu et al. (28). Notably, in the case of maximal activation by co-expression with Ras12V and Lck the substitution of both residues S432 and T442 led to marked decrease of kinase activity (Fig. 3A and C), indicating an additional aspect in the mechanism of A-RAF activation, which could not be identified by activation with EGF alone.

Threonines within the activation segment are necessary for Ras12V/Lck-mediated activation of A-RAF, but dispensable for activation with EGF – Phosphorylation of amino acids within the kinase activation loop has been reported to be necessary for activation of several protein kinases including MEK (52) and ERK (53). The kinase activation loop is highly conserved in all RAF family kinases and lies between the kinase subdomains VII and VIII (amino acids 447–476 in A-RAF, see also Fig. 1). Phosphorylation of the activation segment in B- and C-RAF occurs at the conserved threonine and serine residues: in B-RAF these are T599 and S602 and the corresponding residues in C-RAF are T491 and S494. Although phosphorylation of T599/S602 in B-RAF is sufficient to generate a highly active state of this kinase (29), the corresponding sites in C-RAF require support by additional phosphorylation (30). Notably, no data are available regarding phosphorylation of A-RAF within its activation segment. Therefore we searched for the phosphorylated fragment in mass spectrometry data corresponding to the putative A-RAF activation segment. As shown in Fig. 1B and Table S1 we detected an A-RAF fragment...
(IGDFGLATVKTR) corresponding to the activation segment. Both of the putative phosphorylation sites (T452 and T455) within this peptide were phosphorylated, as revealed by fragmentation analysis (Table 1 and Fig. S2). This finding indicates that both of these threonines may play a regulatory role in A-RAF activation. To test this assumption we prepared A-RAF point mutants in which the threonines 452 and 455 were substituted by alanines. As shown in Fig. 4A and C we did not observe any significant differences in the magnitude of kinase activity for A-RAF-T452A and A-RAF-T455A variants compared to A-RAF WT following EGF stimulation. Surprisingly, the Ras12V/Lck-mediated activation of A-RAF was strongly reduced by substitution of T452 or T455 with alanine (Fig. 4A and C). These results indicate that phosphorylation of A-RAF activation segment does not play a crucial role in the course of EGF-mediated activation; however, it may be necessary for maximal A-RAF activation.

Phosphorylation within the IH-domain regulates positively the activation process of A-RAF – Whereas the initial steps of RAF activation have been investigated in detail, the mechanisms of feedback regulation are only partially understood. Recently, Dougherty et al. (35) discussed the possibility that ERK-induced phosphorylation may be responsible for down regulation of C-RAF kinase activity. These data are in agreement with our contribution (33) in which we identified negative regulation of C-RAF by phosphorylation at serines 296 and 301 within the isoform-specific hinge domain. In the case of B-RAF, phosphorylation of the evolutionarily conserved SPKTP motif located at the C-terminus has been suggested to be involved in negative feedback regulation (54). Regarding feedback regulation of A-RAF no data are available so far. With the exception of the 257SP site in A-RAF that corresponds to the C-RAF 296SP motif, the amino acid sequences of the IH-segments in A- and C-RAF reveal a very low degree of similarity, suggesting that regulation of kinase activity by this segment may occur in an isoform specific way. Indeed, our data obtained by mass spectrometry revealed that A-RAF, similar to C-RAF, is phosphorylated within the IH-domain. We detected several phosphorylated fragments covering the region between amino acids 248 and 288 of A-RAF (see Fig. 1B and Table S1) that correspond to IH-segment. However, in contrast to C-RAF, which has been reported to be phosphorylated at only two sites (S296 and S301), the corresponding A-RAF fragment (248-267) was phosphorylated at all of the seven putative phosphorylation sites. This phosphopeptide was detected only in the Ras12V/Lck activated A-RAF protein. Other peptides derived from this region were partially phosphorylated and were found in both serum- and Ras12V/Lck-stimulated A-RAF samples. In addition we identified two A-RAF specific phosphorylation sites (S272 and S274) next to the IH-region (see also Fig. 1B and Tables 1 and S1). Thus, these findings suggest that A-RAF becomes highly phosphorylated within the IH-region upon prolonged stimulation.

To examine the functional role of the novel phosphorylation sites for A-RAF signaling we prepared single mutations derived from the seven phosphorylation sites present in the GGSDGTPRGSPSPASVSSGR tryptic fragment by replacing each of the six serines and threonine 253 by alanine. As depicted in Fig. 5A and B single mutations at serines 257, 262, and 264 strongly impaired the A-RAF kinase activity upon EGF stimulation. Consistent with these findings Ras/Lck-mediated A-RAF activation was almost completely abolished by the same substitutions (Fig. 5D and E). The replacement of other residues (S250, T253, S259, and S265) by alanine did not cause significant changes of A-RAF activity. Similarly, substitution of serines 272 and 274 by alanine did not impair kinase activity (data not shown).

In this context, we examined also the degree of phosphorylation of other regulatory sites of A-RAF: e.g. serines 582 and 214 within the 14-3-3 binding domains and tyrosines 301/302 within the N-region. Whereas phosphorylation of serine 214 was unchanged for all of the samples, the levels of S582 phosphorylation were considerably reduced in A-RAF-S257A, A-RAF-S262A, and A-RAF-S264A mutants, indicating an interdependence between activity and phosphorylation of the C-terminal 14-3-3 binding site (Fig. 5). Regarding tyrosine phosphorylation, we did not observe any phosphorylation upon EGF stimulation in agreement with original findings (55). In contrast, activation mediated by Ras12V/Lck resulted in efficient tyrosine
phosphorylation of A-RAF WT and substitution mutants with exception of A-RAF-S262A mutant, whose tyrosine phosphorylation was completely abrogated. Although the antibody used here is directed against phosphotyrosine per se, tyrosine phosphorylation induced by co-expression with Ras12V and Lck can be entirely ascribed to tyrosines 301/302 because this antibody did not recognize A-RAF-Y301D-Y302D mutant (see Fig. 6). This finding indicates that A-RAF-S262A mutant is not phosphorylated on tyrosines 301/302 compared to wild type. Notably, it was possible to partially rescue the kinase activity of A-RAF-S262A mutant by additional introduction of aspartic acids in positions Y301 and Y302 that mimics Lck induced phosphorylation in these positions (see Fig. 6).

As three S/TP phosphorylation sites within the IH-segment of A-RAF (253TP, 257SP, and 259SP) and two adjacent SP sites (269 and 274) reveal ERK-directed phosphorylation motifs, we expected that these sites might be involved in ERK-mediated feedback phosphorylation of A-RAF. To this end, we examined activation of A-RAF wild type in the presence and absence of the MEK inhibitor U0126. As demonstrated in Fig. 7 treatment of the cells with U0126 resulted in a considerable reduction of A-RAF activity induced by Ras12V/Lck. Thus, in contrast to C-RAF, in which phosphorylation within the IH-domain contributes to negative feedback regulation, phosphorylation of the IH-domain in A-RAF regulates its kinase activity in a stimulatory manner.

Spatial model reveals charge reversal at the molecular surface of IH-segment of A-RAF upon phosphorylation – The isoform specific IH-segment of A-RAF reveals an unusually high density of putative phosphorylation sites, but in addition also a high number of basic residues (see Fig. 1B). To obtain a spatial image of the distribution of negative charge upon phosphorylation of these sites and to compare this with the distribution of the positive charge of the non-stimulated kinase we performed molecular modeling of the IH-region. The three-dimensional structure of the corresponding peptide comprising residues between S246 and E277 was modeled using Protein Design Model QUANTA2006. Since no homologous structure templates were available, de novo modeling was applied using an extended peptide strand without secondary structure elements as start structure. This starting template was then submitted to molecular dynamics simulations to form a more globular structure. Several structures for the phosphorylated and non-phosphorylated form were simulated to test whether the sequence folds into a common motif, however, consistent with secondary structure prediction neither defined secondary structure elements nor globular structures were observed. Similar results were obtained for phosphorylated and non-phosphorylated forms indicating that phosphorylation did not influence the peptide structure in defined manner. One representative conformer for both the phosphorylated and non-phosphorylated form is illustrated in Fig. 8 (and supplemental Fig. S1) revealing the typical random coil structure. To analyze the changes in charge distribution upon phosphorylation electrostatic potential maps were calculated using the non-linearized Poisson-Bolzmann equation and the software APBS. Upon introduction of multiple phosphorylation sites in positions S250, T253, S257, S259, S262, S264, and S265 we observed a charge-reversal of a mainly positive to a strongly negative electrostatic potential map. This observation forms our current working hypothesis in which we propose that the high accumulation of negative charges may lead to an electrostatic destabilization of protein-membrane interaction resulting in depletion of A-RAF from the plasma membrane upon prolonged stimulation by Ras and Lck. On the other hand, C-RAF, which is lacking this multi-phosphorylation stretch, may behave contrary to A-RAF.

To test this hypothesis we performed analysis of subcellular localization of A- and C-RAF wild type expressed in the presence and absence of Ras12V and Lck. The data illustrated in Fig. 9 indicate that in non-stimulated cells both A- and C-RAF were located preferentially in the cytosolic fraction. In contrast, upon co-expression with Ras and Lck the subcellular distribution of A- and C-RAF was diametrically opposed. Whereas C-RAF has been found to be associated mainly with membranes, A-RAF was highly accumulated in the cytosolic fraction. Thus, these experiments support the view that A- and C-RAF are subject to differential regulation, most probably due to the structural differences within the N- and IH-regions.
DISCUSSION

While much attention has been devoted to the phosphorylation-mediated regulation of B- and C-RAF, with some exception little is known about the role of phosphorylation in the A-RAF activation process.

In the current study we performed a systematic analysis of phosphorylation and regulatory sites of A-RAF by mass spectrometry. The screening of the peptides derived from the serum-activated or Ras12V/Lck-stimulated A-RAF protein revealed a number of phosphorylated fragments that correspond to both, N-terminal regulatory part as well as to the kinase domain (Fig. 1B and Table S1). Most of the phosphorylation sites identified within the kinase domain of A-RAF appeared to be highly conserved suggesting that regulation by phosphorylation of the kinase domain occurs in a similar way for all three RAF isoforms. In contrast the phosphorylation sites that were detected within the N-terminal regulatory part and at the very C-terminus of the protein show high degree of variability. These observations together with the results of our previous study (39) indicate that the unique regulation of each RAF isoform is specified by these variable regions.

C-terminal 14-3-3 binding site is not essential for the Ras/Lck-mediated activation of A-RAF – The participation of 14-3-3 proteins in the activation-deactivation cycle of B- and C-RAF is well established (16,40,56). Dimers of 14-3-3 proteins have been shown to interact with C- and B-RAF by binding to the internal and C-terminal 14-3-3 binding motifs (57). Since the association of RAF with 14-3-3 proteins requires phosphorylation of the serines within the binding motifs, we investigated here by use of mass spectrometry and available phosphospecific antibodies the phosphorylation of the proposed 14-3-3 binding sites in A-RAF. The mass spectrometry data provided partial information. We detected only the tryptic fragment, corresponding to the C-terminal 14-3-3 binding domain (Fig. 1B and Table S1). Data from MS analysis were complemented by use of phosphospecific antibodies. As shown in Fig. 2A, both, the internal and the C-terminal 14-3-3 binding sites of A-RAF proved to be phosphorylated, suggesting that A-RAF may bind 14-3-3 dimers in a similar way, as already described for C- and B-RAF.

The presence of the serines 621 and 729 in C- and B-RAF, respectively has been shown to be crucial for the activation of these kinases (16,40). Regarding the internal 14-3-3 binding domain of B-RAF the substitution of serine 365 by alanine led to a strong increase of kinase activity indicating that the phosphorylation of this 14-3-3 binding site acts highly inhibitory (40). Analogous observations have been made with C-RAF (16,51). The results of the present study performed with Sf9 insect cells show, that the basal kinase activity of the A-RAF-S214A mutant was elevated compared to A-RAF wild type (Fig. 2), suggesting that regulation of A-RAF by the internal 14-3-3 binding site occurs in a similar way as reported for C- and B-RAF. In contrast, the data obtained from the in vitro kinase assay with the A-RAF-S582A mutant revealed, that, with respect to regulation by the C-terminal 14-3-3 binding site, A-RAF behaves differently compared to B- and C-RAF. Whereas the basal activity of A-RAF-S582A was abolished upon substitution of serine 582 by alanine, the presence of the intact C-terminal 14-3-3 binding site was only partially required for kinase stimulation by Ras12V and Lck. Remarkably, fragmentation analysis of the phosphopeptide corresponding to the C-terminal 14-3-3 binding domain revealed an additional phosphorylation at serine 585 (Table 1 and Fig. S2). Phosphorylation of this highly conserved serine has not been observed in B- or C-RAF so far and may be A-RAF specific. The consequence of this phosphorylation may be the prevention of 14-3-3 binding to A-RAF resulting in the extraordinary low extent of A-RAF kinase activity as generally observed.

Phosphorylation of the putative MEK binding site is indispensable for EGF-mediated A-RAF activation, whereas phosphorylation of the activation segment is not – A number of protein kinases have been shown to be regulated by phosphorylation within the kinase domain. In the case of RAF proteins, phosphorylation of the MEK binding domain and the activating segment has been reported to be necessary for kinase activity of C- and B-RAF (28-30). Although the entire coupling area between C-RAF and MEK has not yet been determined it has recently been
reported that serine 471 in C-RAF (and the corresponding residue serine 579 in B-RAF) is critical for its kinase activity and for binding to MEK (28). These phosphorylation sites are located within the region that is highly conserved among mammalian RAF isoforms and RAF from other species (see also Fig. 1B). In our study we detected an A-RAF phosphopeptide, located between residues 432–444, that includes the putative MEK binding domain and contains two potential phosphorylation sites: S432 and T442 (Fig. 1B and Table S1). Using the functional substitution analysis we show that S432 is strictly required for A-RAF kinase activity. In contrast, the dependence of A-RAF activity on T442 was restricted to activation by Ras and Lck only. These data suggest that, whereas phosphorylation of the single S432 is necessary and sufficient for EGF-mediated A-RAF activation, the maximal activation of A-RAF kinase requires phosphorylation of both residues, S432 and T442. Our observations are partially in agreement with results recently obtained by Zhu et al. (28) for C- and B-RAF. They reported that S471 of C-RAF and the corresponding residue of B-RAF (S579) are critical for activation of these isoforms by growth factors, whereas substitution of T481 and T588 in C- and B-RAF, respectively, do not have much effect on their kinase activities. Unfortunately, this group did not investigate the importance of the S471/T481 of C-RAF and the corresponding sites in B-RAF for the maximal activation of these isoforms by Ras12V and Lck. Thus, the role of the T481 and T589 in the functioning of C- and B-RAF isoforms, respectively, is not completely elucidated. Additionally, Zhu et al. (28) report that substitution of S471 by alanine in C-RAF almost completely diminished association of this RAF isoform with MEK, whereas the C-RAF-T481A mutant bound MEK as well as wild type, suggesting that the S471, but not T481 may serve as the MEK binding site for C-RAF. However, the authors did not mention, whether the C-RAF used in the pull-down assay has been stimulated. Therefore, the question whether T481 may be involved in the MEK binding to C-RAF remains to be investigated. In the case of maximal A-RAF activation, it is possible that the introduction of negative charge within the N-region caused by Ras/Lck may change the contact area between A-RAF and MEK, thus, making T442 also necessary for transduction of the signals from RAF to MEK. However, other possibilities such as interaction of T442 with some RAF activating components should also be considered.

In contrast to the MEK binding site, which serves as docking platform for substrate, phosphorylation of the activation segment has been reported to induce intra-molecular rearrangement allowing RAF kinase to fold into the active conformation (3). As phosphorylation of the residues within the activation segment has been established for C- and B-RAF only, in the present study we investigated the role of the activation segment in the regulation of A-RAF kinase activity. Identification of the phosphorylated A-RAF tryptic fragment IGDFGLATVKTR containing phosphate residues at both T452 and T455 (Fig. 1B and Table S1) suggests that, similar to B- and C-RAF, the activation segment of A-RAF kinase may be involved in the activation process. Confirming this assumption the results from the functional mutation study showed that depending on stimulation conditions either both residues or neither are necessary for A-RAF activation (Fig. 4). The results of kinase activity assay have revealed that EGF-mediated stimulation of A-RAF does not require phosphorylation on threonines 452 and 455 within the activation segment, whereas maximal activation by co-operation of Ras and Lck strongly depends on phosphorylation of these residues. In part these data are in accordance with results obtained for B- and C-RAF activation. Zhang et al. (29) have shown convincingly that phosphorylation of T599 and S602 is essential for B-RAF activation. In contrast, the role of phosphorylation within the activation segment of C-RAF is controversially discussed. Whereas Barnard et al. (58) reported that phosphorylation of the activation loop plays no significant role in C-RAF activation, the functional mutation study performed by Chong et al. (30) revealed that the activation of the T491A or S494A mutants was diminished, demonstrating that these residues play indeed a regulatory role in C-RAF activation. Structural studies using the crystal structure of the B-RAF kinase domain (59) provided more insight into how RAF proteins may be regulated by the activation segment. There is a hydrophobic interaction between the glycine-rich
loop and the activation segment that traps RAF in an inactive conformation. Phosphorylation of the threonine within the activation segment (T599 in B-RAF) is thought to disrupt this interaction releasing the activation segment and reorienting critical residues into the correct position for catalysis (3,59). Our results suggest that this activation model can also be applied to A-RAF. Furthermore, considering the fact that A-RAF kinase activity depends on phosphorylation of the activation segment only in the case of Ras/Lck-mediated stimulation, we suggest that the negative charge at the N-region caused by Ras/Lck is a prerequisite for phosphorylation of activation segment indicating a functional inter-dependence between phosphorylation of S299/Y302 and T452/T455 in A-RAF. Similar considerations were reported by Chong et al. (30) for C-RAF. They have shown that double acidic residue substitutions at either S338/Y341 or T491/S494 in C-RAF were insufficient to confer constitutive activity. In contrast, when the S338D/Y341D mutation was combined with T491E/S494D mutation, C-RAF became constitutively active. From these observations Chong et al. (30) concluded that the requirement for phosphorylation at S338 and Y341 in C-RAF is likely to be the reason for insufficient activation of C-RAF by the T491E/S494D substitution.

Substitution of the valine 600 by glutamic acid within the activation segment of B-RAF (B-RAF-V600E) mimicking the conformational change normally induced by phosphorylation of T599 and S602 (60) leads to a highly activated B-RAF kinase that could not be further activated by Ras12V. Notably, in about 70% of malignant melanomas the highly active B-RAF-V600E mutant was detected (61,62). Whether mutation of the corresponding A-RAF valine 453 to glutamic acid in the activation segment elevates the activity of A-RAF remains to be investigated. Emuss et al. (63) recently showed that C-RAF is not activated by V492E mutation presumably due to the fact that the C-RAF N-region lacks negative charge in non-stimulated cells.

**ERK-mediated feedback phosphorylation is suggested to participate in A-RAF activation** – Regulation of kinase activity by phosphorylation within the non-conserved region, which is located between the CR2 and kinase domain, appears to be isoform specific. For example, phosphorylation of the residues S428 and T440, that are unique for B-RAF, has been reported to regulate negatively its kinase activity (64). Furthermore, in our previous study we demonstrated that the non-conserved residues within the N-region determine partially the basal and the inducible activity of RAF isoforms (39). In the case of C-RAF a domain between serine 289 and serine 301 has been reported to participate in regulation of C-RAF activity by phosphorylation of residues S289, S296 and S301 (33-35). In the present study we identified using mass spectrometry technique several novel A-RAF phosphorylation sites within the non-conserved region between the CR2 and the kinase domain. Data obtained by MS (see Fig. 1B and Table S1) provided eight phosphorylated fragments covering the region between the residues G248 and K288. Importantly, one of these fragments has been found to be phosphorylated at seven putative phosphorylation sites and was detected only in the Ras12V/Lck-stimulated A-RAF, whereas other fragments carrying one, two or four phosphate residues have been detected in both serum-activated and Ras12V/Lck-stimulated proteins (Fig. 1B and Table S1). These data suggest that the prolonged stimulation of A-RAF kinase may be accompanied with hyper-phosphorylation of regulatory IH-domain. The role of the intermediate phosphorylations and their progression during activation remains to be elucidated. Also the activity state of such differentially phosphorylated intermediates is a matter of speculation.

On the other hand, we show here that single point mutations within the IH-domain generated by substitution of serines or threonine with alanine resulted in some cases in reduction of induced A-RAF kinase activity (Fig. 5 A, B, D, and E). In contrast to A-RAF mutants S250A, T253A, S259A, and S265A whose catalytic activity was not significantly diminished, substitutions of S257, S262, and S264 led to almost complete inactivation of A-RAF kinase suggesting that these three residues are crucial for its regulation. Considering these observations it is tempting to speculate that phosphorylation of S257, S262 or S264 might be a prerequisite for phosphorylation of further sites within the IH-domain. If that would be the case, then replacement of each of these crucial sites would partially or even completely abrogate the hyper-
phosphorylation of the IH-domain. However, at present we can only speculate about the hierarchy of phosphorylation events within this domain.

Furthermore, by use of phosphospecific antibodies we observed a relationship between different regulatory phosphorylation sites. Whereas phosphorylation of S214 was unchanged in all IH-mutants, the level of pS582 was diminished in the samples that displayed reduced kinase activity (Fig. 5 A, C, D, and F). The homologous serine in C-RAF (S621) has been proposed to function as an auto-phosphorylation site (16). A similar consideration may apply to serine 582 of A-RAF. In this case, the reduced S582 phosphorylation of A-RAF IH-point mutants would be the result of their low kinase activity. However, other possibilities such as dephosphorylation by phosphatases should also be considered. Although tyrosine phosphorylation of several A-RAF IH-mutants was slightly diminished, only the A-RAF-S262A mutant almost completely abrogated YY301/302 phosphorylation (Fig. 5 D and G), indicating that interplay between these two phosphorylation sites may exist. Interestingly, introduction of the additional Y301D/Y302D substitution into the A-RAF-S262A mutant only partially rescued kinase activity (Fig. 6). The partial rescue suggests that phosphorylation at the S262 of the IH-domain may be required for activating tyrosine phosphorylation within the N-region. The current phosphopeptide screening revealed that A-RAF WT is not only phosphorylated at Y301/Y302, but also at two additional tyrosines: Y24 and Y419 (Fig. 1B and Table S1). Whereas Y24 is A-RAF specific, Y419 is highly conserved among all RAF proteins. Y419 is equivalent to Y538 of D-RAF (Drosophila RAF), which together with Y510 (Y391 in human A-RAF) was recently discussed by Xia et al. (65) as targets for Src-mediated phosphorylation. However, experimental results confirmed only Y510, but not Y538 as a proper target for phosphorylation by Src. Whether A-RAF Y24 and Y419 are substrates for human Src family kinases and whether phosphorylation of these sites is impaired by S262A substitution remains to be investigated.

From seven phosphorylation sites within the A-RAF 248-267 tryptic fragment three are part of the ERK-specific S/TP phosphorylation motifs (Fig. 1B). Based on this observation ERK-mediated feedback phosphorylation of T253, S257, and S259 in the IH-domain of A-RAF should be considered. ERK-mediated feedback phosphorylation has already been found to modulate kinase activity of B- and C-RAF (34,35,54). However, entirely different motifs have been described to be involved in this process. While in B-RAF the evolutionarily conserved SPKTP motif at the C-terminus has been made responsible for feedback regulation (54) C-RAF has the sequence SPRLP in this location. Although a SP site is present in this fragment, other regions that are not present in B-RAF have been proposed to be involved in feedback regulation of C-RAF. The positions S289, S296, and S301 in C-RAF are targets of ERK proline-directed kinase phosphorylation (Fig. 1B). The consequences of phosphorylation at these positions for C-RAF activity were described as negative by Dougherty et al. and Hekman et al. and positive by Balan et al. (33-35). Perhaps some of the differences in results relate to the fact that Dougherty et al. and Balan et al. did not use single site mutants for their evaluation. As it stands, definitive conclusions regarding the role of ERK-mediated phosphorylation in C-RAF require additional investigation. Regarding feedback regulation of A-RAF our present results demonstrate that the putative ERK-induced phosphorylation of T253/S257/S259 is involved in the positive regulation of its kinase activity (Fig. 7). Of these three sites, serine 257 showed the most pronounced effect on A-RAF catalytic activity. Whereas T253 and S259 do not possess counterparts in C-RAF, S257 of A-RAF is analogous to S296 of C-RAF. However, although this residue is conserved in both isoforms, the relative position of S257 in A-RAF protein may differ considerably. The sequence surrounding the S296 in C-RAF is six amino acids shorter than the corresponding region in A-RAF (see Fig. 1B). The suggestion that ERK-mediated feedback phosphorylation of T253/S257/S259 may positively regulate A-RAF activity is further supported by the results obtained using MEK inhibitor U0126. A-RAF wild type isolated from the U0126-treated cells displayed considerably decreased catalytic activity compared to the protein derived from the untreated cells (Fig. 7) suggesting that ERK-mediated feedback regulation is required for effective activation of A-RAF. This
raises the possibility that ERK regulates A-RAF activation by feedback phosphorylation at these SP motifs. In addition, adjacent to the T253/S257/S259 sites two other SP motifs are present in A-RAF, i.e. 269SP and 274SP and should also be considered as putative ERK phosphorylation sites. One of them (S274) has been indeed identified in its phosphorylated state (Table 1 and Fig. S2).

Molecular modeling of the IH-Segment suggests a "switch-of-charge" mechanism for A-RAF regulation — Recruitment of RAF kinases to the plasma membrane was initially proposed to be mediated by Ras proteins via interaction with the RAF-RBD. However, data published by our group and others showed that Ras-independent association with membrane lipids is involved in the RAF translocation pathway as well (13,66,67). Interaction of B- and C-RAF with negatively charged phospholipids has been documented by several studies. Besides the ability to interact with RAS and 14-3-3 proteins C-RAF-CRD has been shown to associate with phosphatidylserine at the plasma membrane (68,69). In addition, a phosphatidic acid binding domain has been identified within the CR3 of C-RAF kinase (13,14,66). This domain is highly conserved in all three RAF isoforms and comprises a RKTR motif, which is rich in positively charged amino acids. In the case of A-RAF, lysine 50 and arginine 52 within the RBD as well as several basic residues within the region between amino acids 200 and 606 have been suggested to be implicated in binding of negatively charged phospholipids such as phosphatidic acid and phosphorylated phosphoinositides (70). However, although several basic residues of A-RAF were included in this study, Johnson et al. (70) did not examine the role of positively charged residues within the IH-segment. Due to the fact that the IH-region comprises six basic residues the modeling of the non-phosphorylated form of the IH-region, revealed a relatively high density of positive charge that may facilitate A-RAF association with plasma membrane before and during activation. On the other hand, in the case that all of the seven phosphorylation sites located within the tryptic fragment 248-267 were considered to carry a phosphate residue (as suggested by the MS findings presented in Fig. 1B and Table S1) a high accumulation of negative charge at the surface of the molecule emerged in the three dimensional reconstruction (Fig. 8 and S1). This observation prompts us to speculate, that the complete phosphorylation of the IH-region may result in depletion of A-RAF from membranes. Indeed, data obtained by subcellular fractionation revealed that, in contrast to C-RAF, which was enriched preferentially in the membrane fraction, the majority of A-RAF was located in the cytosolic fraction upon stimulation with Ras12V/Lck (Fig. 9). This observation is consistent with data of Yuryev and Wennogle (71) and Mazurek et al. (72), who reported that A-RAF regulates a number of cytosolic proteins.

Although all three RAF family members (A-, B-, and C-RAF) overlap in their regulation and choice of substrate there are significant differences in their activation/deactivation profiles and in the degree of ERK activation (73). Based on results presented here and data published by Wixler et al. (73) we suggest a model for A-RAF activation that differs from models proposed for B- and C-RAF. According to this model the rapid A-RAF stimulation that is of short duration (1-2 min) takes place at the plasma membrane, where Ras GTPases and Src family kinases are located. The association of inactive A-RAF with the plasma membrane may be facilitated by the high density of positive charge at the IH-region as demonstrated by molecular modeling (see Fig. 8 and S1). Activating phosphorylations such as phosphorylation of the N-region upon growth factor stimulation would moderately enhance the catalytic activity of A-RAF in this location. Sustained activation would be delayed relative to B- and C-RAF that first generate active ERK needed for feedback phosphorylation of A-RAF in the IH region. Such a delay may facilitate the function of A-RAF in endocytosis that has recently become evident (74, Nekhoroshkova et al. in preparation). We propose further that the stimulatory phosphorylation of residues S257 and/or S262, and/or S264 within the IH-domain may have a dual effect. On the one hand, it facilitates and accelerates activation of A-RAF at the plasma or endocytic membranes. On the other hand, it may also recruit phosphorylation of further residues within the IH-domain, resulting in hyper-phosphorylation of this region. Upon multiple phosphorylation events at the IH-region catalyzed by Ras and Lck we propose a switch of
charge in the IH-region that may be responsible for dissociation of A-RAF from plasma membrane. Nevertheless, the exact fate and physiological function of the hyper-phosphorylated A-RAF fraction remains unknown. For C-RAF a negative modulation of membrane localization by hyper-phosphorylation was discussed (35,75). However, the proposed inactivation mechanism of C-RAF differs considerably from the model discussed here. Contrary to A-RAF, displacement of C-RAF from the plasma membranes requires phosphorylation of six serine residues distributed across the whole molecule. Moreover, four of these sites are C-RAF specific and are not present in A-RAF. Therefore, the most important message of our modeling and fractionation studies is that A-RAF behaves distinct than C-RAF upon hyper-phosphorylation. Nevertheless, to obtain more insight into the complex activation cycle of A-RAF development of phosphospecific antibodies directed against the regulatory sites within the IH-domain is necessary. The generation of these antibodies is in progress.

References

1. Rapp, U. R., Goldsborough, M. D., Mark, G. E., Bonner, T. I., Groffen, J., Reynolds, F. H., Jr., and Stephenson, J. R. (1983) Proc Natl Acad Sci U S A 80, 4218-4222.
2. Dhillon, A. S., and Kolch, W. (2002) Arch Biochem Biophys 404, 3-9
3. Wellbrock, C., Karasarides, M., and Marais, R. (2004) Nat Rev Mol Cell Biol 5, 875-885
4. Rapp, U. R., Gotz, R., and Albert, S. (2006) Cancer Cell 9, 9-12
5. Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A., and Ecker, J. R. (1993) Cell 72, 427-441
6. Han, M., Golden, A., Han, Y., and Sternberg, P. W. (1993) Nature 363, 133-140
7. Mark, G. E., MacIntyre, R. J., Digan, M. E., Ambrosio, L., and Perrimon, N. (1987) Mol Cell Biol 7, 2134-2140
8. Bonner, T. I., Kerby, S. B., Sutrave, P., Gunnell, M. A., Mark, G., and Rapp, U. R. (1985) Mol Cell Biol 5, 1400-1407
9. Daum, G., Eisenmann-Tappe, I., Fries, H. W., Troppmair, J., Rapp, U. R. (1994) Trends Biochem Sci 19, 474-480.
10. Huleihel, M., Goldsborough, M., Cleveland, J., Gunnell, M., Bonner, T., and Rapp, U. R. (1986) Mol Cell Biol 6, 2655-2662
11. Ikawa, S., Fukui, M., Ueyama, T., Tamaoki, N., Yamamoto, T., and Toyoshima, K. (1988) Mol Cell Biol 8, 2651-2654
12. Hagemann, C., and Rapp, U. R. (1999) Exp Cell Res 253, 34-46
13. Hekman, M., Hamm, H., Villar, A. V., Bader, B., Kuhlmann, J., Nickel, J., and Rapp, U. R. (2002) J Biol Chem 277, 24090-24102.
14. Rizzo, M. A., Kraft, C. A., Watkins, S. C., Levitan, E. S., and Romero, G. (2001) J Biol Chem 276, 34928-34933
15. Garnett, M. J., Rana, S., Paterson, H., Barford, D., and Marais, R. (2005) Mol Cell 20, 963-969
16. Hekman, M., Wiese, S., Metz, R., Albert, S., Troppmair, J., Nickel, J., Sendtner, M., and Rapp, U. R. (2004) J Biol Chem 279, 14074-14086
17. Rushworth, L. K., Hindley, A. D., O'Neil, E., and Kolch, W. (2006) Mol Cell Biol 26, 2262-2272
18. Weber, C. K., Slupsky, J. R., Kalmes, H. A., and Rapp, U. R. (2001) Cancer Res 61, 3595-3598.
19. Fischer, A., Hekman, M., Kuhlmann, J., Rubio, I., Wiese, S., and Rapp, U. R. (2007) J Biol Chem 282, 26503-26516
20. Morrison, D. K., Heidecker, G., Rapp, U. R., and Copeland, T. D. (1993) J Biol Chem 268, 17309-17316.
21. Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J., and Cantley, L. C. (1997) Cell 91, 961-971.
22. Reusch, H. P., Zimmermann, S., Schaefer, M., Paul, M., and Moelling, K. (2001) J Biol Chem 276, 33630-33637
23. Moelling, K., Schad, K., Bosse, M., Zimmermann, S., and Schweneker, M. (2002) J Biol Chem 277, 31099-31106
24. Dhillon, A. S., Pollock, C., Steen, H., Shaw, P. E., Mischak, H., and Kolch, W. (2002) Mol Cell Biol 22, 3237-3246
25. Yip-Schneider, M. T., Miao, W., Lin, A., Barnard, D. S., Tzivion, G., and Marshall, M. S. (2000) Biochem J 351, 151-159
26. Mischak, H., Seitz, T., Janosch, P., Eulitz, M., Steen, H., Schellmer, M., Philipp, A., and Kolch, W. (1996) Mol Cell Biol 16, 5409-5418
27. Zhu, J., Balan, V., Bronisz, A., Balan, K., Sun, H., Leicht, D. T., Luo, Z., Qin, J., Avruch, J., and Tzivion, G. (2005) Mol Biol Cell 16, 4733-4744
28. Zhang, B. H., and Guan, K. L. (2000) Embo J 19, 5429-5439
29. Chong, H., Lee, J., and Guan, K. L. (2001) Embo J 20, 3716-3727
30. Stephens, R. M., Sithanandam, G., Copeland, T. D., Kaplan, D. R., Rapp, U. R., and Morrison, D. K. (1992) Mol Cell Biol 12, 3733-3742
31. Xing, H. R., and Kolesnick, R. (2001) J Biol Chem 276, 9733-9741
32. Hekman, M., Fischer, A., Wennogle, L. P., Wang, Y. K., Campbell, S. L., and Rapp, U. R. (2005) FEBS Lett 579, 464-468
33. Hekman, M., Fischer, A., Wennogle, L. P., Wang, Y. K., Campbell, S. L., and Rapp, U. R. (2005) FEBS Lett 579, 464-468
34. Balan, V., Leicht, D. T., Zhu, J., Balan, K., Kaplun, A., Singh-Gupta, V., Qin, J., Ruan, H., Comb, M. J., and Tzivion, G. (2006) Mol Cell Biol 17, 1141-1153
35. Dougherty, M. K., Muller, J., Ritt, D. A., Zhou, M., Zhou, X. Z., Copeland, T. D., Conrads, T. P., Veenstra, T. D., Lu, K. P., and Morrison, D. K. (2005) Mol Cell 17, 215-224
36. Diaz, B., Barnard, D., Filson, A., MacDonald, S., King, A., and Marshall, M. (1997) Mol Cell Biol 17, 4509-4516
37. Marais, R., Light, Y., Paterson, H. F., Mason, C. S., and Marshall, C. J. (1997) J Biol Chem 272, 4378-4383
38. Mason, C. S., Springer, C. J., Cooper, R. G., Superti-Furga, G., Marshall, C. J., and Marais, R. (1999) Embo J 18, 2137-2148
39. Baljuls, A., Mueller, T., Drexler, H. C., Hekman, M., and Rapp, U. R. (2007) J Biol Chem 282, 26575-26590
40. Brummer, T., Martin, P., Herzog, S., Misawa, Y., Daly, R. J., and Reth, M. (2006) Oncogene 25, 6262-6276
41. Rost, B., and Liu, J. (2003) Nucleic Acids Res 31, 3300-3304
42. Cuff, J. A., Clamp, M. E., Siddiqui, A. S., Finlay, M., and Barton, G. J. (1998) Bioinformatics 14, 892-893
43. Baker, N. A., Sept, D., Joseph, S., Holst, M. J., and McCammon, J. A. (2001) Proc Natl Acad Sci U S A 98, 10037-10041
44. Homeyer, N., Horn, A. H., Lanig, H., and Sticht, H. (2006) J Mol Model 12, 281-289
45. Neuhoff, V., Arolf, N., Taube, D., and Ehrhardt, W. (1988) Electrophoresis 9, 255-262
46. Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T., and Mann, M. (1996) Nature 379, 466-469
47. Reinders, J., Wagner, K., Zahedi, R. P., Stojanovski, D., Eyrich, B., van der Laan, M., Rehling, P., Sickmann, A., Pfanner, N., and Meisenger, C. (2007) Mol Cell Proteomics 6, 1896-1906
48. Zahedi, R. P., Lewandowski, U., Wiesner, J., Wirtelkamp, S., Moebius, J., Schutz, C., Walter, U., Gambaryan, S., and Sickmann, A. (2008) J Proteome Res 7, 526-534
49. Tzivion, G., Luo, Z., and Avruch, J. (1998) Nature 394, 88-92
50. Suen, K. L., Bustelo, X. R., and Barbacid, M. (1995) Oncogene 11, 825-831.
51. Kubicek, M., Pacher, M., Abraham, D., Podar, K., Eulitz, M., and Baccarini, M. (2002) J Biol Chem 277, 7913-7919.
52. Alessi, D. R., Saito, Y., Campbell, D. G., Cohen, P., Sithanandam, G., Rapp, U., Ashworth, A., Marshall, C. J., and Cowley, S. (1994) Embo J 13, 1610-1619
53. Payne, D. M., Rosomando, A. J., Martino, P., Erickson, A. K., Her, J. H., Shabanowitz, J., Hunt, D. F., Weber, M. J., and Sturgill, T. W. (1991) *Embo J* **10**, 885-892.

54. Brummer, T., Naegele, H., Reth, M., and Misawa, Y. (2003) *Oncogene* **22**, 8823-8834.

55. App, H., Hazan, R., Zilberstein, A., Ullrich, A., Schlessinger, J., and Rapp, U. (1991) *Mol Cell Biol* **11**, 913-919.

56. Tzivion, G., and Avruch, J. (2002) *J Biol Chem* **277**, 3061-3067.

57. Dougherty, M. K., and Morrison, D. K. (2004) *J Cell Sci* **117**, 1875-1884.

58. Tzivion, G., and Avruch, J. (2002) *J Biol Chem* **277**, 3061-3064.

59. Brummer, T., Naegele, H., Reth, M., and Misawa, Y. (2003) *Oncogene* **22**, 8823-8834.

60. Barnard, D., Diaz, B., Clawson, D., and Marshall, M. (1998) *Oncogene* **17**, 1539-1547.

61. Wan, P. T., Garnett, M. J., Roe, S. M., Lee, S., Niculescu-Duvaz, D., Good, V. M., Jones, C. M., Marshall, C. J., Springer, C. J., Barford, D., and Marais, R. (2004) *Cell* **116**, 855-867.

62. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. (2002) *Nature* **417**, 949-954.

63. Improta-Brears, T., Ghosh, S., and Bell, R. M. (1999) *Mol Cell Biochem* **198**, 171-178.

64. Johnson, L. M., James, K. M., Chamberlain, M. D., and Anderson, D. H. (2005) *Biochemistry* **44**, 3432-3440.

65. Yuryev, A., and Wennogle, L. P. (2003) *Genomics* **81**, 112-125.

66. Wartmann, M., Hofer, P., Turowski, P., Saltiel, A. R., and Hynes, N. E. (1997) *J Biol Chem* **272**, 3915-3923.

FOOTNOTES

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The abbreviations used are: MS, mass spectrometry; Sf9, insect cell line derived from pupal ovarian tissue of *Spodoptera frugiperda*; COS7, African Green Monkey SV40-transfected kidney fibroblast cell line; FCS, fetal calf serum; DMEM, Dulbecco’s modified Eagle’s medium; NP-40, Nonidet P-40; RBD, Ras-binding domain; CRD, cysteine-rich domain; IH-segment, Isoform-specific Hinge segment; EGF, epidermal growth factor; MEK, mitogen-activated protein kinase kinase; ERK, extracellular-signal regulated kinase; pTyr, phosphotyrosine, WT, wild type; α, anti; IB, immunoblotting assay; IP, immunoprecipitation.

**FIGURE LEGENDS**

**Fig. 1.** A, schematic presentation of human A-, B-, and C-RAF kinases with their regulatory domains. B, sequence alignment of human A-, B-, and C-RAF depicting representative tryptic phosphopeptides of A-RAF obtained by mass spectrometry analysis. For the detailed list of all the identified phosphopeptide see Table S1. The conserved regions CR1, CR2, and CR3 are highlighted in salmon pink. The sequences of tryptic phosphopeptides of A-RAF are underlined in green. The molecular weight (MW) of the tryptic fragments and the number of the phosphate residues (P) are indicated. The putative phosphorylation sites of A-RAF are highlighted in green and their positions within the sequence are indicated by numbers. The corresponding conserved sites in B- and C-RAF are highlighted in grey.

**Fig. 2.** A-RAF activity is regulated by phosphorylation of both internal and C-terminal 14-3-3 binding sites. A, Western blot analysis of A-RAF kinase activity and phosphorylation degree of 14-3-3 binding sites. A-RAF wild type (WT) and A-RAF substitution mutants modified at the C-terminal (S582A) and internal (S214A) 14-3-3 binding sites were expressed in Sf9 insect cells in the presence and absence of Ras12V and Lck. The cells were lysed using detergent containing buffer as described in *Experimental Procedures*. Subsequently, coupled kinase assay using recombinant MEK and ERK as substrates has been carried out, and the extents of kinase activities were monitored by anti-pERK antibody. To monitor the phosphorylation degree of A-RAF in the positions S214 and S582 the C-RAF phosphospecific antibodies anti-pS259 and anti-pS621 (see also Hekman et al. (16)) were used. Due to the homology of the corresponding epitopes these antibodies detect both RAF isoforms with comparable affinity. B, quantification of A-RAF kinase activity. Representative blots from the A were quantified by optical densitometry. The quantification results are expressed in terms of fold activation, where 1-fold activity represents the amount of activity determined for A-RAF wild type under non-stimulating conditions. Average values derived from the data of three independent experiments were used for quantification. Expression efficiency of H-Ras12V and Lck was determined using anti-H-Ras and anti-Lck antibodies, respectively. IB, immunoblots.

**Fig. 3.** Serine 432 is critical for catalytic activity of A-RAF in vivo. A, Western blot analysis of A-RAF kinase activity. C-terminal Myc-tagged A-RAF wild type (WT) and substitution mutants (S432A and T442A) were expressed in COS7 cells. Stimulation was performed either by treatment of the cells with EGF (100 ng/ml) for 5 min or by co-expression with H-Ras12V and Lck. Cells were lysed as described in *Experimental Procedures* and A-RAF proteins were immunoprecipitated (IP) by use of an anti-Myc antibody. Subsequently, kinase activity was determined in the presence of recombinant MEK and ERK as substrates. ERK phosphorylation was detected by phosphospecific anti-ERK antibody. B and C, quantification of A-RAF kinase activity upon activation with EGF or co-expression with Ras12V/Lck, respectively. Representative blots from the A were quantified by optical densitometry. The quantification results are expressed in terms of fold activation, where 1-fold activity represents the amount of activity determined for A-RAF wild type. Average values derived from the data of three independent experiments were used for quantification. Expression efficiency of H-Ras12V and Lck was determined using anti-H-Ras and anti-Lck antibodies, respectively. Actin immunodetection was used as a loading control. IB, immunoblots.
Fig. 4. Threonins 452 and 455 located within the activation segment are not involved in the EGF-mediated stimulation of A-RAF. A, Western blot analysis of A-RAF kinase activity. C-terminal Myc-tagged A-RAF wild type (WT) and substitution mutants (T452A and T455A) were expressed in COS7 cells. Stimulation was performed either by treatment of the cells with EGF (100 ng/ml) for 5 min or by co-expression with H-Ras12V and Lck. Cells were lysed as described in Experimental Procedures and A-RAF proteins were immunoprecipitated (IP) by use of an anti-Myc antibody. Subsequently, coupled kinase assay using recombinant MEK and ERK as substrates has been carried out and the extents of kinase activities were monitored by anti-pERK antibody. B and C, quantification of A-RAF kinase activity upon activation with EGF or co-expression with Ras12V/Lck, respectively. Representative blots from the A were quantified by optical densitometry. The quantification results are expressed in terms of fold phosphorylation, where 1-fold activity represents the amount of activity determined for A-RAF wild type. Average values derived from the data of three independent experiments were used for quantification. Expression efficiency of H-Ras12V and Lck was determined using anti-H-Ras and anti-Lck antibodies, respectively. Actin immunodetection was used as a loading control. IB, immunoblots.

Fig. 5. Phosphorylation sites within the IH-segment of A-RAF regulate its catalytic activity in a stimulatory manner. COS7 cells were transfected with A-RAF wild type (WT) or A-RAF substitution mutants as indicated. Following stimulation the cells were lysed as described in Experimental Procedures and A-RAF proteins were immunoprecipitated (IP) by use of an anti-Myc antibody. Subsequently, kinase activities were measured using recombinant MEK and ERK as substrates. ERK phosphorylation was detected by phosphospecific anti-ERK antibody. To monitor the phosphorylation degree of A-RAF in the positions S214 and S582 the C-RAF phosphospecific antibodies anti-pS259 and anti-pS621 (see also Hekman et al. (16)) were used. Due to the homology of the corresponding epitops these antibodies detect both RAF isoforms with comparable affinity. Tyrosine phosphorylation of A-RAF was determined by anti-phosphotyrosine (pTyr) antibody 4G10. A, Western blot analysis of A-RAF kinase activity and phosphorylation degree of S214 and S582 upon stimulation with EGF (100 ng/ml) for 5 min. B, quantification of A-RAF kinase activity upon activation with EGF. The quantification results are expressed in terms of fold activation, where 1-fold activity represents the amount of activity determined for A-RAF wild type. Average values derived from the data of five independent experiments were used for quantification. C, quantification of serine 582 phosphorylation upon activation with EGF. The quantification results are expressed in terms of fold phosphorylation, where 1-fold of phosphorylation represents the amount of phosphorylation determined for A-RAF wild type. D, Western blot analysis of A-RAF kinase activity and phosphorylation degree of S214, S582 and Y301/Y302 upon co-expression with Ras12V and Lck. E, quantification of A-RAF kinase activity upon co-expression with Ras12V/Lck. The quantification results are expressed in terms of fold activation, where 1-fold activity represents the amount of activity determined for A-RAF wild type. Average values derived from the data of three independent experiments were used for quantification. F and G, quantification of serine 582 and tyrosines 301/302 phosphorylation, respectively, upon co-expression with Ras12V/Lck. The quantification results are expressed in terms of fold phosphorylation, where 1-fold of phosphorylation represents the amount of phosphorylation determined for A-RAF wild type. Expression efficiency of H-Ras12V and Lck was determined using anti-H-Ras and anti-Lck antibodies, respectively. Actin immunodetection was used as a loading control. IB, immunoblots.

Fig. 6. Introduction of Y301D/Y302D substitution into the A-RAF-S262A mutant rescues partially its kinase activity. C-terminally Myc-tagged A-RAF wild type (WT) and A-RAF substitution mutants (S262A, Y301D/Y302D, and S262A/Y301D/Y302D) were expressed in COS7 cells in presence of H-Ras12V and Lck. Cell lysis, immunoprecipitation (IP) with anti-Myc antibody, and kinase activity measurements were carried out as described in Fig. 5. Tyrosine phosphorylation of A-RAF was determined by anti-phosphotyrosine (pTyr) antibody 4G10. Expression efficiency of H-Ras12V and Lck was determined using anti-H-Ras and anti-Lck antibodies, respectively. Actin immunodetection was used as a loading control. IB, immunoblots.
Fig. 7. Abolishment of ERK-mediated feedback regulation by treatment with MEK inhibitor U0126 results in reduction of A-RAF kinase activity. C-terminally Myc-tagged A-RAF wild type (WT) was expressed in COS7 cells in the presence of H-Ras12V and Lck. Prior to lysis the cells were treated with MEK inhibitor U0126 (10 µM) for 12h. Immunoprecipitation (IP) with anti-Myc antibody and kinase activity measurements were carried out as described in Fig. 5. A, Western blot analysis of A-RAF kinase activity. B, quantification of A-RAF kinase activity. The quantification results are expressed in terms of fold activation, where 1-fold activity represents the amount of activity determined for A-RAF wild type derived from the untreated cells. Average values derived from the data of two independent experiments were used for quantification. Expression efficiency of H-Ras12V and Lck was determined using anti-H-Ras and anti-Lck antibodies, respectively. Detection of phosphorylated ERK in whole cell lysates was used to verify the efficiency of MEK inhibition by U0126. ERK immunodetection was used as a loading control. IB, immunoblots.

Fig. 8. Charge reversal at the molecular surface of IH-segment in A-RAF upon phosphorylation. A, stereoview of the electrostatic potential map of the A-RAF fragment (residues between S246 and E277) containing the IQ region with residues Ser250, Thr253, Ser257, Ser259, Ser262, Ser264, and Ser265 being phosphorylated. The peptide region adopts a random coil structure. One representative of five calculated structures has been chosen for quantification of the electrostatic potential. Isocontours for -0.5 kT/e (red) and +0.5 kT/e (blue) are shown. The region comprising the phospho-serine and -threonine residues shows a highly negative potential besides the presence of several positively charged amino acids. B, stereoview of the electrostatic potential of the IH-region of A-RAF in its non-phosphorylated form. In contrast to A various basic residues cause a mainly positively charged potential, thus, suggesting a switch of charge upon phosphorylation.

Fig. 9. Subcellular fractionation study reveals opposite distribution of activated A- and C-RAF. A, Western blot analysis of subcellular distribution of activated and non-activated A-RAF wild type (WT) (left panel) and C-RAF wild type (WT) (right panel). COS7 cells were transfected with Myc-tagged A- and C-RAF wild type in the presence and absence of H-Ras12V and Lck. After 24 h of serum starvation, cytoplasmic, membrane, nuclear, and cytoskeletal fractions were collected according to the protocol of the ProteoExtract subcellular proteome extraction kit. Fractionation control: anti-M2PK immunodetection for cytosolic fraction, anti-Histon 3 immunodetection for nuclear fraction, and anti-vimentin immunodetection for cytoskeletal fraction. Expression of H-Ras12V and Lck was detected using anti-H-Ras and anti-Lck antibodies, respectively. IB, immunoblots. B, quantification of A- and C-RAF protein distribution between cytosolic and membrane fractions. Data from two independent experiments were quantified by optical densitometry. Values represent % ratio of RAF protein in each fraction relative to the total RAF protein detected in both cytosolic and membrane fractions together.
Table 1: Phosphorylation sites specified by fragmentation analysis using nano-LC-MS/MS approach. P-site, phosphorylation site; z, precursor charge; m/z, mass-to-charge ratio; Mr(expt), experimental mass; Mr(calc), theoretical mass; Delta, mass deviation Mr(expt)-Mr(calc). All MascotTM result pages of the manually validated phosphopeptide spectra from this table are attached in supplementary data (Fig. S2).

| Position | Peptide sequence    | P-site       | m/z   | z | Mr(expt) | Mr(calc) | Delta | Mass analyzer |
|----------|---------------------|--------------|-------|---|----------|----------|-------|---------------|
| 17-28    | AVGTVKVpYLPNK       | Y24          | 685,00| 2 | 1367,98  | 1367,72  | 0,26  | LTQ XL       |
| 256-267  | GpSPSPASVSSGR       | S257         | 584,75| 2 | 1167,49  | 1167,49  | 0,00  | QstarElite    |
| 256-267  | GpSPSPApSVSSGR      | S257, S262   | 624,83| 2 | 1247,64  | 1247,46  | 0,19  | LTQ XL       |
| 256-267  | GpSPSPASVpSSGR      | S257, S264   | 625,35| 2 | 1248,68  | 1247,46  | 1,22  | LTQ XL       |
| 269-281  | SPHlpSKpSPAEPQER    | S272, S274   | 556,92| 3 | 1667,72  | 1667,68  | 0,04  | LTQ XL       |
| 316-326  | IGlFGSTGTVFR        | T318         | 611,29| 2 | 1220,56  | 1220,56  | 0,00  | QstarElite    |
| 445-456  | IGDFGLApTVKpTR      | T452, T455   | 719,21| 2 | 1436,40  | 1436,65  | -0,25 | LTQ XL       |
| 544-551  | RLLpSDCLK           | S547         | 502,78| 2 | 1003,55  | 1003,55  | 0,00  | QstarElite    |
| 580-588  | SAPSEPSLHR          | S582         | 532,23| 2 | 1062,44  | 1062,45  | -0,01 | QstarElite    |
| 580-588  | SAPSEpPLHR          | S582, S585   | 572,20| 2 | 1142,40  | 1142,40  | -0,02 | QstarElite    |
Figure 2

A.

|          | WT | S582A | S214A |
|----------|----|-------|-------|
| H-Ras12V| -  | +     | -     |
| Lck      | -  | +     | -     |

**α-pERK IB**

**α-ERK IB**

**α-A-RAF IB**

**α-pS214 IB**

**α-pS582 IB**

**α-Lck IB**

**α-H-Ras IB**

*in vitro kinase assay*

*whole cell lysate*

B.

![Bar chart](http://www.jbc.org/Downloaded from)

**A-RAF kinase activity [fold WT]**

|          | - H-Ras12V/Lck | + H-Ras12V/Lck |
|----------|----------------|----------------|
| A-RAF:   | WT             | S582A          |
|          | S214A          |                |

[Downloaded from](http://www.jbc.org/Downloaded from)
Figure 3

A.

|                  | + 5 min EGF | + H-Ras12V + Lck |
|------------------|-------------|------------------|
| A-RAF:           | WT          | WT               |
|                  | S432A       | S432A            |
|                  | T442A       | T442A            |
| α-pERK IB        |             |                  |
| α-ERK IB         |             |                  |
| α-Myc IB         |             |                  |
| α-Lck IB         |             |                  |
| α-H-Ras IB       |             |                  |
| α-Actin IB       |             |                  |

IP: Myc + in vitro kinase assay
whole cell lysate

B.

C.

A-RAF kinase activity (fold WT)

A-RAF: WT S432A T442A

A-RAF: WT S432A T442A

A-RAF: WT S432A T442A

+ H-Ras12V + Lck

+ 5 min EGF
Figure 4

A.

+ 5 min EGF

+ H-Ras12V + Lck

A-RAF: WT T452A T455A

α-pERK IB

α-ERK IB

α-Myc IB

α-Lck IB

α-H-Ras IB

α-Actin IB

IP: Myc + in vitro kinase assay

whole cell lysate

B.

C.

A-RAF kinase activity (fold WT)

A-RAF: WT T452A T455A

+ 5 min EGF

+ H-Ras12V + Lck

A-RAF: WT T452A T455A
Figure 5

D.

+ H-Ras12V + Lck

| A-RAF:  | WT   | S250A | T253A | S257A | S259A | S262A | S264A | S265A |
|--------|------|-------|-------|-------|-------|-------|-------|-------|
| α-pERK IB |      |       |       |       |       |       |       |       |
| α-ERK IB  |      |       |       |       |       |       |       |       |
| α-Myc IB  |      |       |       |       |       |       |       |       |
| α-pS582 IB |     |       |       |       |       |       |       |       |
| α-pS214 IB |     |       |       |       |       |       |       |       |
| α-pTyr IB |      |       |       |       |       |       |       |       |
| α-Lck IB  |      |       |       |       |       |       |       |       |
| α-H-Ras IB |     |       |       |       |       |       |       |       |
| α-Actin IB |     |       |       |       |       |       |       |       |

*in vitro* kinase assay

E.

![Graph showing A-RAF kinase activity](image)

A-RAF kinase activity [fold WT]

| A-RAF:  | WT   | S250A | T253A | S257A | S259A | S262A | S264A | S265A |
|--------|------|-------|-------|-------|-------|-------|-------|-------|
|        |      |       |       |       |       |       |       |       |

+ H-Ras12V + Lck
Figure 5

F. Phosphorylation of A-RAF serine 582 (fold WT) in the presence of + H-Ras12V + Lck.

G. Phosphorylation of A-RAF tyrosines 301/302 (fold WT) in the presence of + H-Ras12V + Lck.
Figure 6

\[ + \text{H-Ras12V} + \text{Lck} \]

A-RAF: WT, S262A, Y301D, Y302D

\( \alpha \)-pERK IB
\( \alpha \)-ERK IB
\( \alpha \)-Myc IB
\( \alpha \)-pTyr IB
\( \alpha \)-Lck IB
\( \alpha \)-H-Ras IB
\( \alpha \)-Actin IB

*in vitro* kinase assay

IP: Myc

whole cell lysate
### Figure 7

**A.**

| Treatment | U0126 | DMSO | H-Ras12V | Lck |
|-----------|-------|------|----------|-----|
| Condition | -     | +    | +        | +   |

**IB:**

- α-pERK IB
- α-ERK IB
- α-Myc IB
- α-Lck IB
- α-H-Ras IB
- α-pERK IB
- α-ERK IB

**IP:** Myc + *in vitro* kinase assay

**Whole cell lysate**

**B.**

| Treatment         | Kinase activity of A-RAF (fold untreated) |
|-------------------|------------------------------------------|
| Untreated         | 1                                        |
| + DMSO            | 0.9 ± 0.1                                |
| + U0126           | 0.4 ± 0.2                                |
Figure 9

A.

| Protein            | A-RAF(WT) | C-RAF(WT) |
|--------------------|-----------|-----------|
| α-Myc IB           |           |           |
| α-Lck IB           |           |           |
| α-H-Ras IB         |           |           |
| α-M2PK IB          |           |           |
| α-Histon 3 IB      |           |           |
| α-Vimentin IB      |           |           |

- Ras12V/Lck + Ras12V/Lck

B.

Subcellular distribution of A-RAF(WT) and C-RAF(WT) [%]

- A-RAF
  - Ras12V/Lck
  - + Ras12V/Lck

- C-RAF
  - Ras12V/Lck
  - + Ras12V/Lck

- cytoplasm
- membranes
Positive regulation of A-RAF by phosphorylation of isoform-specific hinge segment and identification of novel phosphorylation sites
Angela Baljuls, Werner Schmitz, Thomas Mueller, Rene P. Zahedi, Albert Sickmann, Mirko Hekman and Ulf R. Rapp

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