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Raf Kinase Activation of Adenylyl Cyclases: Isoform-Selective Regulation

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ABSTRACT

Adenylyl cyclases (AC), a family of enzymes that catalyze the synthesis of cyclic AMP, are critical regulators of cellular functions. The activity of adenylyl cyclase is stimulated by a range of hormone receptors, primarily via interactions with G-proteins; however, recently we identified an alternate mechanism by which growth factors sensitize adenylyl cyclase activation. We suggested that this mechanism might involve a Raf kinase-mediated serine phosphorylation of adenylyl cyclase. However, the direct involvement of a specific form of Raf kinase is yet to be demonstrated. Furthermore, whether this mechanism is generalized to other isoforms of adenylyl cyclase is unknown. In human embryonic kidney 293 cells, we now demonstrate that in reconstitution studies, c-Raf kinase can mediate phosphorylation of AC VI. Furthermore, AC VI coimmunoprecipitates with c-Raf. Raf kinase-dependent regulation of adenylyl cyclase VI is dependent on the integrity of Ser750 in the fourth intracellular loop of the enzyme and Ser603/Ser608 in the C1b region of the molecule. To examine how generalized this effect is, we studied representative isoforms of the major subfamilies of adenylyl cyclase viz., AC I, AC II, and AC V. Raf kinase-dependent sensitization/ phosphorylation of adenylyl cyclases is common to AC VI, AC V, and AC II isoforms but not AC I. In aggregate, these studies indicate that Raf kinase associates with adenylyl cyclases. Furthermore, Raf kinase regulation of adenylyl cyclase is isoform-selective. These functional interactions (as well as the physical association) between adenylyl cyclases and Raf kinases suggest an important but previously unrecognized interaction between these two key regulatory enzymes.

Adenylyl cyclase is a critical effector enzyme, linking receptor activation with regulation of cellular functions. The activity of the enzyme adenylyl cyclase (AC), which catalyzes the generation of cyclic AMP from ATP, is itself regulated by both extracellular stimuli via activation of G-protein-coupled receptors and intracellular stimuli. Regulation of adenylyl cyclase activation is primarily by G-protein interactions or calcium-calmodulin interactions (Cooper et al., 1995; Hanoune and Defer, 2001; Patel et al., 2001); however, alternate mechanisms of adenylyl cyclase activation involving regulation of the phosphorylation state of the enzyme have also been identified.

Serine phosphorylation of the enzyme regulates the function of several adenylyl cyclase isoforms (Premont et al., 1992; Zimmermann and Taussig, 1996; Bol et al., 1997). This effect has been reported to be both inhibitory (the effect of cAMP-dependent protein kinase-mediated phosphorylation) (Premont et al., 1992) and stimulatory (the effect of protein kinase C-mediated phosphorylation) (Zimmermann and Taussig, 1996; Bol et al., 1997). The effects of protein kinase C-mediated adenylyl cyclase phosphorylation have been reported to be isoform-specific (Yoshimura and Cooper, 1993).

Activation and regulation of adenylyl cyclases has been viewed traditionally in the context of a serial signaling cascade linking G-protein-coupled receptor activation to regulation. Likewise, activation of tyrosine kinases (the predominant mechanism by which growth factors regulate intracellular function) has been viewed traditionally as part of a parallel and separate signaling cascade. Recent studies have suggested multiple points of convergence of these pathways—including at the level of regulation of adenylyl cyclase function.

Adenylyl cyclase activity is regulated by the activation of both receptor-linked and nonreceptor-linked tyrosine kinases (Heath and Terrar, 1994; Jap et al., 1994; Valiquette et al., 1995; Graves et al., 1996; Poppleton et al., 1996; Shih and Malbon, 1998). In general, the effect of tyrosine kinase acti-

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Q.D. and R.G. contributed equally to this work.

ABBREVIATIONS: AC, adenylyl cyclase; PCR, polymerase chain reaction; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; MEKK, mitogen-activated protein kinase kinase kinase.

vation is to increase adenylyl cyclase activity, although inhibitory effects also have been described. Studies from our laboratory have demonstrated tyrosine kinase-dependent sensitization of adenylyl cyclase catalytic activity, independent of changes in either receptor or heterotrimeric G-protein activity (Feldman, 1993; Tan et al., 1995, 1999). We suggested recently (based on pharmacological probes and the use of a dominant-negative mutant of c-Raf) that the growth factor/tyrosine kinase-mediated sensitization of adenylyl cyclase type VI involved Raf kinase-mediated serine phosphorylation (Tan et al., 2001). This pathway has been implicated in the superactivation of adenylyl cyclase after long-term opiate exposure (Varga et al., 2002).

Our studies to date have demonstrated that tyrosine kinase-dependent serine phosphorylation of adenylyl cyclase VI enhances the activity of the adenylyl cyclase enzyme (Tan et al., 2001); however, the molecular mechanism of this effect is unclear. In particular, the specific form of Raf kinase that mediated adenylyl cyclase phosphorylation is unknown. In addition, the identity of the individual serine residues that are phosphorylated to mediate sensitization of adenylyl cyclase activity remain to be determined. Furthermore, it is unknown whether this interaction is unique for AC VI or whether tyrosine kinase-mediated regulation is a phenomenon common to other adenylyl cyclase isoforms.

In those previous studies, we generated several serine to alanine mutations of residues located in both the first catalytic loop subdomain (C1b region—mutating serine molecules at both amino acid residues 603 and 608) and in the fourth intracellular loop (IC4 region—mutating four serine molecules at amino acid residues 744, 746, 750, and 754) of adenylyl cyclase (Tan et al., 2001). These mutants demonstrated significantly reduced vanadate-stimulated phosphorylation and sensitization of adenylyl cyclase compared with wild-type AC VI (Tan et al., 2001); however, the identity of the single amino acid residue(s) critical in Raf kinase-mediated phosphorylation was not determined.

Based on these uncertainties, we sought to determine: 1) whether adenylyl cyclase could serve as a substrate for c-Raf kinase, 2) the specific amino acid residues in adenylyl cyclase critical for Raf kinase-mediated regulation, and 3) whether Raf kinase-mediated regulation of AC VI was generalized among other subfamilies of adenylyl cyclase isoforms. In the present studies, we demonstrate the interaction between c-Raf kinase and adenylyl cyclase in a reconstituted system and have identified the single amino acids critical for this effect. Furthermore, we demonstrate that this interaction is adenylyl cyclase isoform-selective.

Materials and Methods

Mutagenesis of FLAG Adenylyl Cyclase VI. Six single serine (S) to alanine (A) mutants (S603A, S608A, S744A, S746A, S750A, and S754A) were generated via overlapping extension PCR mutagenesis (see Table 1) of our previously described FLAG-tagged AC VI (Tan et al., 2001). In brief, two independent PCR reactions were carried out to generate a pair of overlapping PCR products. The first reaction used an upstream sense-mutagenizing oligonucleotides paired with a downstream antisense harboring a unique restriction enzyme digestion site. The second reaction used upstream sense oligonucleotides harboring a unique restriction enzyme digestion site paired with a downstream antisense-mutagenizing primer. Each PCR product was resolved by agarose electrophoresis, followed by the Qiaquick PCR purification (QIAGEN, Valencia, CA). Equivalent amounts (50 ng) of PCR products from first pair PCR were amplified using extreme sense and antisense primers. The resulting PCR were purified and double digested with the terminal restriction enzyme, then cloned into pcDNA3-flag AC VI that had been digested with identical restriction enzyme digestion, positive clones were sequenced to verify the right open reading frame and right mutation.

Epitope Tagging of Adenylyl Cyclase Isoforms I, II, and V. FLAG-tagged AC V, AC II, and AC I were generated by PCR using pCMV5-AC V, pCDM8.1-AC II, and pCMV5-AC I as templates using the following primers: Flag AC V, forward AC V (5′-CCC AAG CTT GCC ACC ATG TGC AGC AGC AGC AGC GCC TGG-3′) and reverse AC V (5′-GC TCT AGA CTA CTT ATC GTC GTC ATC CTT GTA ATC ACT GAG CGG GGG CCC ACC GTT GAG-3′); Flag AC II, forward AC II (5′-CCC AAG CTT GCC ACC ATG GAT TAC AAG GAT GAC

TABLE 1
Oligonucleotides used to generate site-specific serine to alanine AC VI mutants

Candidate Serine Residues for Mutation	Primer Name	Primer Sequence
603	S603A_fp	5′-TGGGTTCCTGACGTGCCTTCGCCCGGACCAAGGACTCTAAGGCA-3′
	S603A_rp	5′-TGCCTTAGAGTCCTTGGTCCGGGCGAAGGCACGGTCAGGAACCCA-3′
608	S608A_fp	5′-GCCCATCTGTCGGAATGCCTTGGCGTCCTTGGTCCGGGAGAAGGC-3′
	S608A_rp	5′-GCCCATCTGTCGGAATGCCTTGGCGTCCTTGGTCCGGGAGAAGGC-3′
744	S744A_fp	5′-CCCAACGCCCTGCAGCGCCTGGCCCGCAGTATCGTCCGCTCACGG-3′
	S744A_rp	5′-CCGTGAGCGGACGATACTGCGGGCCAGGCGCTGCAGGGCGTTGGG-3′
746	S746A_fp	5′-GCCCTGCAGCGCCTGTCCCGCGCCATCGTCCGCTCACGGGTGCAC-3′
	S746A_rp	5′- GTGCACCCGTGAGCGGACGATGGCGCGGGACAGGCGCTGCAGGGC-3′
750	S750A_fp	5′-CTGTCCCGCAGTATCGTCCGCGCCCGGGTGCACAGCACGGCTGTT-3′
	S750A_rp	5′-AACAGCCGTGCTGTGCACCCGGGCGCGGACGATACTGCGGGACAG-3′
754	S754A_fp	5′-ATCGTCCGCTCACGGGTGCACGC CACGGCTGTTGGAGTCTTCTCG-3′
	S754A_rp	5′-CGAGAAGACTCCAACAGCCGTGGCGTGCACCCGTGAGCGGACGAT-3′
Restriction enzyme sites		
NheI	NheI_fp	5′-CACCGGCTAGCGCAG-3′
XhoI	XhoI_fp	5′-AGGGAGGATCTCGAGAAGAAG-3′
	XhoI_rp	5′-CTTCTTCTCGAGATCCTCCCT-3′
XbaI	XbaI_rp	5′-TCT CTAGAC TAA CTG CTG GGG C CC CCA TT-3′
	Round 1	Round 2
Cassette	PCR1	PCR2
S603A	NheI_fp, S603A_rp	S603A_fp, XhoI_rp NheI_fp, XhoI_rp
S608A	NheI_fp, S608A_rp	S608A_fp, XhoI_rp NheI_fp, XhoI_rp
S744A	XhoI_fp, S744A_rp	S774A_fp, XbaI_rp XhoI_fp, XbaI_rp
S746A	XhoI_fp, S746A_rp	S746A_fp, XbaI_rp XhoI_fp, XbaI_rp
S750A	XhoI_fp, S750A_rp	S750A_fp, XbaI_rp XhoI_fp, XbaI_rp
S754A	XhoI_fp, S754A_rp	S854A_fp, XbaI_rp XhoI_fp, XbaI_rp

GAC GAT AAG CGG CGG CGC CGC TAC CTG CGG-3′) and reverse AC II(5′-GC TCT AGA TCA GGA TGC CAA GTT GCT CTG-3′); and Flag AC I, forward AC I (5′-CCC AAG CTT GCC ACC ATG GAT TAC AAG GAT GAC GAC GAT AAG GCC GCG GGG GCG CCG CGC GGC CGA GGC-3′) and reverse AC I (5′-GC TCT AGA CTA AGC CTC CTT CCC AGA GGC TCC AGG-3′). PCR fragments were sequenced and digested with HindIII/XbaI and cloned into a pcDNA3 vector digested with identical enzyme.

Raf Constructs. Both pcDNA3-flag-c-Raf and pcDNA3-b-Raf were provided by Dr. Ron Taussig (University of Texas Southwestern Medical Center at Dallas); pcDNA c-Raf (non-FLAG-tagged) was constructed by PCR using pcDNA3-flag-c-Raf as a template. Forward primer 5′-GGGGATCC(BamHI)GCCGCCATGGAGCACATACAGG-GAGCT TGG-3′ and reverse primer 5′-GCTCTAGA(XbaI) CTAGAA-GACAGGCAGCCTCGG GGA-3′, PCR fragment was digested with BamHI and XbaI and cloned into pcDNA3 vector digested with identical enzymes.

Cell Culture and Transfections. HEK 293 cells were grown at 37°C, 5% CO_2 in minimum essential medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum. HEK 293 cells were transiently transfected with pcDNA3 containing adenylyl cyclase VI single point mutants or isoforms I, II, V, or VI cDNA. For adenylyl cyclase-Raf interaction experiments, HEK cells were cotransfected with vector (sham) or adenylyl cyclase isoforms and/or Raf kinases (see below).

Assessment of Adenylyl Cyclase Activity. Adenylyl activity was assessed by the conversion of [α-^{32}P]ATP to [^{32}P]cAMP as described previously (Tan et al., 2001). Maximal catalytic activity was assessed with forskolin (10 μM). Tyrosine kinase-dependent activation of adenylyl cyclase was assessed with forskolin (10 μM) plus vanadate (300 μM).

Immunoprecipitation and Western Blotting. Transfected cells were lysed in buffer (20 mM Tris, pH 8.0, 1% Nonidet P-40, 0.1% SDS, 140 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, 1 μg antipain, 1 μg leupeptin, and 1 μg pepstatin A). Cell extracts were incubated with anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO) for 2 h at 4°C on a rocker platform. The resulting immunoprecipitation complexes were resolved on SDS-PAGE, blotted electrophoretically onto Immun-Blot polyvinylidene fluoride membrane (Bio-Rad, Hercules CA). The membrane were blocked with 5% skim milk and incubated either with an anti-adenylyl cyclase polyclonal antibody, generated against a 14-amino acid peptide of the C-terminal region of adenylyl cyclase, common to all cloned adenylyl cyclase isoforms [AC comm, 1:3000] (Pieroni et al., 1995; Tan et al., 2001), anti-b-Raf (1:300) or anti-c-Raf (1:300 both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody and were detected by chemiluminescence as described by the manufacturer's protocol (PerkinElmer Life and Analytical Sciences, Boston, MA).

Adenylyl Cyclase Phosphorylation Assays. Assays of FLAG-AC isoform phosphorylation were performed as described previously (Tan et al., 2001). In brief, HEK 293 cells transfected with FLAG-adenylyl cyclase isoforms I, II, V, or VI were permeabilized and resuspended in Hanks' balanced salt solution, pH 7.4, with 33 mM HEPES, 1.25 mM EDTA, and 5 mM $MgSO_4$ to a final concentration of 1 mg/ml. Permeabilized cells were incubated with 0.1 mCi/ml [γ-^{32}P]ATP (PerkinElmer Life and Analytical Sciences) in the presence or absence of 300 μM vanadate for 30 min at 30°C. Adenylyl cyclase isoforms were immunoprecipitated and immune complexes were separated by SDS-PAGE. Phosphorylated proteins were visualized via autoradiography. To assess the ability of c-Raf or b-Raf to phosphorylate adenylyl cyclase in purified protein assays, enriched membrane fractions from AC VI-expressing Sf9 cells were incubated in 40 μl of a solution of 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ATP, 5 μCi of [γ-^{32}P] ATP with or without active c-Raf (1U) or active b-Raf (3U; both obtained from Upstate Cell Signaling Solutions, Lake Placid, NY) for 30 min at 30°C. Proteins were resolved by SDS-PAGE. Phosphorylated adenylyl cyclase was visualized via autoradiography.

Results

Identification of a Functional Interaction between Raf Kinase and Adenylyl Cyclase. To determine whether Raf-dependent serine phosphorylation of adenylyl cyclase VI might occur in vitro in a reconstituted system, we assessed the effect of coincubation of purified active c-Raf on the phosphorylation of AC VI (in purified membrane lysates). The addition of active c-Raf significantly enhanced phosphorylation of adenylyl cyclase VI (Fig. 1A). It is noteworthy that the addition of active b-Raf to enriched AC VI membranes did not enhance the phosphorylation of AC VI (data not shown).

To examine whether the c-Raf kinase-mediated phosphorylation of adenylyl cyclase might represent a direct interaction between Raf kinase and AC VI, we determined whether Raf kinase proteins are coimmunoprecipitated with AC VI. c-Raf was identified as a 74-kDa protein immunoblotted using a c-Raf-specific antibody that migrated identically with a protein identified in whole cell lysates from c-Raf-positive control cells. c-Raf coimmunoprecipitated with AC VI in cotransfected HEK 293 cells (Fig. 1B).

To determine whether AC VI also could be shown to associate with other Raf kinases, we performed coimmunoprecipitation studies between AC VI and either a dominant-negative mutant of c-Raf [DN-Raf, as described previously (Tan et al., 2001)] or with b-Raf. Comparable expression of both c-Raf and dn-Raf was evident in membranes immunoblotted with the anti-c-Raf antibody (Fig. 1B). b-Raf was identified as a protein of 85 kDa immunoblotted using a b-Raf-specific antibody, which migrated identically with a protein identified in whole cell lysates from b-Raf-positive control cells. With coexpression of AC VI with these Raf kinases, both DN-c-Raf (Fig. 1B) and b-Raf (Fig. 1C) coimmunoprecipitated with adenylyl cyclase VI. These data support the hypothesis of a direct protein-protein interaction between Raf kinases and AC VI. Notably, only the interaction/association of adenylyl cyclase VI with c-Raf kinase (but not b-Raf nor DN-c-Raf) mediated the AC VI phosphorylation and the enhancement of adenylyl cyclase activity. Thus, a Raf-kinase/adenylyl cyclase physical association may be required but is clearly not sufficient for Raf kinase-mediated phosphorylation/sensitization of adenylyl cyclase activity.

Identification of the Specific Amino Acid Residues Critical in the Raf Kinase-Mediated Adenylyl Cyclase Phosphorylation. Our previous studies had identified the domains involved in the serine phosphorylation of adenylyl cyclase but not the specific residues. To determine which of the individual serine residues are involved in mediating the tyrosine kinase-mediated Raf-dependent serine phosphorylation of AC VI, we generated single serine to alanine mutants in the regions previously identified as involved in the serine phosphorylation and sensitization of adenylyl cyclase VI. All of the AC VI mutants and AC VI were similarly expressed and detected in whole cell lysates (Fig. 2) and in membrane fractions but not in cytosolic fractions (data not shown). We have previously shown that the phosphorylation state of FLAG-AC VI was significantly increased by a tyrosine kinase-dependent mechanism after vanadate stimulation (Tan et al., 2001). Therefore, we examined the ability of vanadate to increase phosphorylation of both FLAG-AC VI and FLAG-tagged mutants of AC VI. Vanadate increased the phosphorylation state of a 132-kDa protein species corresponding to

FLAG-AC VI and all of the various single serine > alanine mutants of AC VI (Fig. 2, A and C) *except* the 750A mutant, which did not demonstrate increased phosphorylation after vanadate stimulation (Fig. 2A). An additional 145-kDa phosphorylated protein was also detected, however, that was also apparent in sham-transfected cells. Protein expression of AC VI and all mutants was verified by Western blotting and was not altered after vanadate stimulation (Fig. 2, B and D). To determine whether the expression of the various mutants of AC VI resulted in altered adenylyl cyclase activation, we assessed adenylyl cyclase activation after both forskolin- and vanadate-stimulated conditions in FLAG-AC VI and mutants of AC VI. Paralleling the results from phosphorylation studies, transfection of wild-type FLAG-AC VI as well as all of the mutants *except the 750A mutant* significantly enhanced vanadate-stimulated adenylyl cyclase activation compared with sham-transfected cells (Table 2 and Fig. 3). It is noteworthy that expression of the 750A mutant did not result in any

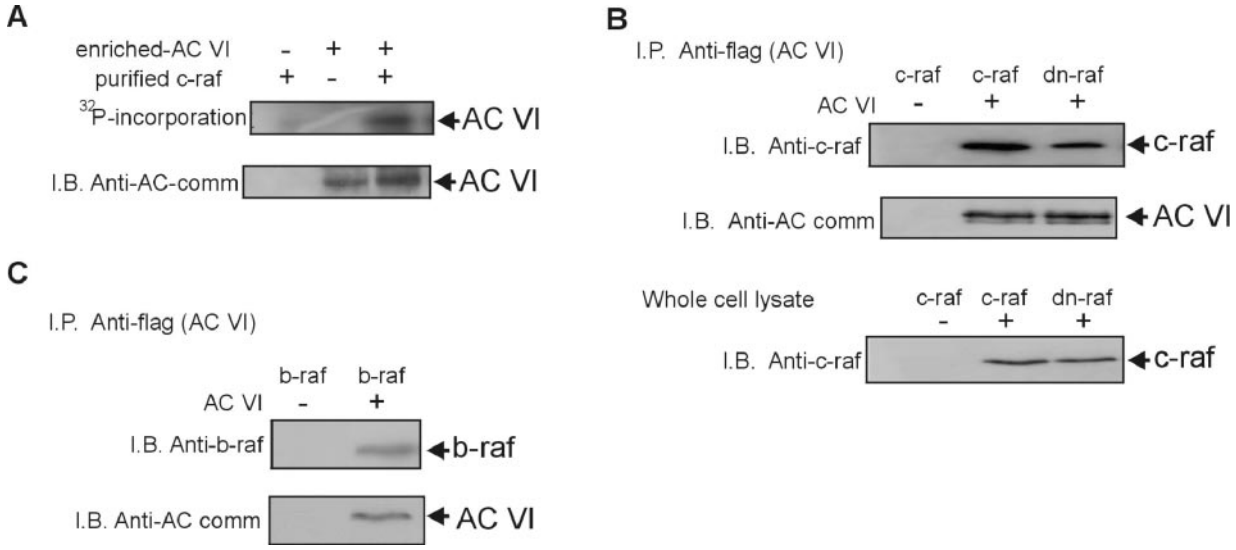

Fig. 1. Interaction and association of Raf-1 kinase with adenylyl cyclase VI. Phosphorylation of adenylyl cyclase VI by active c-Raf kinase (A). Adenylyl cyclase VI enriched membrane fractions were incubated in kinase buffer with or without active c-Raf. The reaction was initiated by the addition of [γ-^{32}P]ATP. Proteins were separated by SDS-PAGE. The phosphorylation of adenylyl cyclase VI was visualized by autoradiography. The expression of AC VI in enriched membrane fractions from AC VI-infected Sf9 cells is demonstrated after immunoblotting with anti-AC comm. Coimmunoprecipitation of adenylyl cyclase isoform VI with c-Raf and DN-c-Raf (B) and b-Raf (C). HEK 293 cells coexpressing FLAG-tagged adenylyl cyclase VI and either c-Raf or b-Raf kinase were lysed, immunoprecipitated with anti-FLAG affinity beads, and separated by SDS-PAGE. The presence of either c-Raf or b-Raf protein in anti-FLAG immune complexes was detected by Western blotting with either c-Raf- or b-Raf-specific antibodies. The comparable expression of c-Raf and DN-Raf is demonstrated in whole cell lysates after immunoblotting with an anti-c-Raf antibody (B). Immunoreactive adenylyl cyclase VI after immunoprecipitation with anti-FLAG antibody and immunoblotting with anti-adenylyl cyclase antibody (AC comm) is depicted in B and C. The blots depicted are representative of three separate experiments performed under identical conditions.

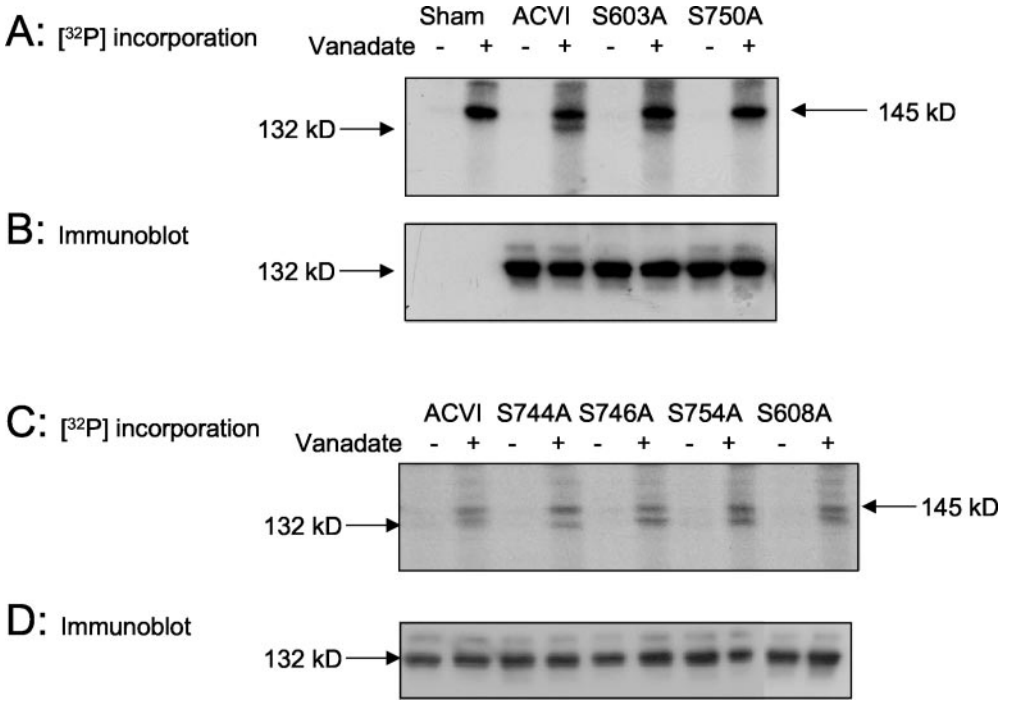

Fig. 2. Assessment of phosphorylation and expression after vanadate treatment. HEK 293 cells transfected with either FLAG-AC VI or its mutants were permeabilized and labeled with [γ-^{32}P]ATP. Cells were lysed and adenylyl cyclases were immunoprecipitated with anti-FLAG antibodies. Effect of vanadate (300 μM) treatment on the phosphorylation state of adenylyl cyclase VI and single serine > alanine mutants (A and C). Adenylyl cyclase VI and single serine > alanine mutants were identified as 132-kDa proteins, not seen in sham-transfected cells. A nonspecific (i.e., present in sham-transfected cells) vanadate-sensitive 145-kDa peptide was also identified. Lack of effect of vanadate treatment on expression of adenylyl cyclase VI and single mutants (B and D). Cell lysates were immunoprecipitated with anti-FLAG antibodies as above. Expression of adenylyl cyclase VI and mutants were identified as 132-kDa peptides (not present in sham-transfected cells) with anti-adenylyl cyclase antibody (AC comm). Adenylyl cyclase expression was not altered by vanadate treatment.

significant increases either in vanadate-stimulated adenylyl cyclase activation or in forskolin-stimulated activation compared with sham-transfected HEK 293 cells (and enzyme activity in the 750A mutant was significantly decreased compared with wild-type FLAG-AC VI; Table 2 and Fig. 3).

Characterization of the Isoform Specificity of the Adenylyl Cyclase-Raf Kinase Interaction. To determine whether this interaction between AC VI and Raf kinases was unique to AC VI or generalized to other isoforms of adenylyl cyclase, we assessed the Raf kinase/adenylyl cyclase interaction in HEK 293 cells expressing various adenylyl cyclase isoforms. This included: 1) AC V, which shares extensive homology with AC VI and is the other member of the AC VI subfamily; 2) AC II, a member of the subfamily that includes AC IV and AC VII; and 3) AC I, a member of the subfamily including AC III and AC VIII (Patel et al., 2001). The expression of the individual isoforms of adenylyl cyclase was verified by Western blotting (Figs. 4A, inset of Figs. 5 and 6, B and D). The adenylyl cyclase isoforms migrated to estimated molecular masses (Fig. 4A; AC I = 115 kDa, AC II = 110 kDa, AC V = 110 kDa, and AC VI = 132 kDa) comparable with the range of molecular masses described for adenylyl cyclases (Ludwig and Seuwen, 2002). With transfection of all of the adenylyl cyclase isoforms studied, forskolin-stimulated activity in AC-transfected HEK 293 cells was significantly increased compared with sham-transfected cells (Table 3). Enhancement of vanadate-mediated adenylyl cyclase activation, however (compared with sham-transfected cells) (Table 3 and Fig. 5), was only apparent with expression of AC isoforms II, V, and VI but not with expression of isoform I. Paralleling these findings, vanadate mediated the phosphorylation of FLAG-tagged adenylyl cyclase isoforms II, V, and VI but not the phosphorylation of isoform I (Fig. 4B)

To determine whether isoform-specific differences in Raf kinase-mediated enhancement of adenylyl cyclase activity and Raf kinase-mediated phosphorylation of adenylyl cyclases might be caused by isoform-specific differences in the physical interaction between adenylyl cyclases and Raf kinases, we assessed the structural and functional association of these adenylyl cyclase isoforms with b-Raf and c-Raf kinases; however, for all adenylyl cyclase isoforms tested, we demonstrated an association with both b-Raf and c-Raf kinases using coimmunoprecipitation techniques (Fig. 6).

TABLE 2

Forskolin- and vanadate-stimulated adenylyl cyclase activity in HEK 293-transfected cells

Adenylyl cyclase activity was expressed as picomoles per minute per milligrams of protein. Vanadate-stimulated activity was expressed as the increase in activity above forskolin-stimulated activity in picomoles per minute per milligrams of protein. The data represent the mean ± S.E.M. from four to seven separate experiments.

Transfected Plasmid	Forskolin-Stimulated Activity	Vanadate-Stimulated Activity
	pmol/min/mg	
Sham	1043 ± 115	771 ± 105
AC VI	1452 ± 139*	1561 ± 171*
603A	1659 ± 57*	1731 ± 464*
608A	2265 ± 282*†	2598 ± 882*†
744A	1394 ± 98*	1785 ± 520*
746A	1289 ± 71*	1755 ± 516*
750A	1104 ± 70	795 ± 160
754A	1455 ± 170*	1580 ± 244*

* $p < 0.05$ versus sham-transfected cells.
† $p < 0.05$ versus AC VI-transfected cells.

Discussion

In previous studies from our laboratory, we have demonstrated that two groups of serine residues located in either the IC4 and C1b domain of adenylyl cyclase VI play a role in the tyrosine kinase-dependent phosphorylation of adenylyl cyclase VI (Tan et al., 2001). In the present study, we demonstrate: 1) that c-Raf kinase (but not b-Raf kinase) phosphorylates AC VI in an enzyme-enriched reconstituted system, 2) that a direct physical interaction can be

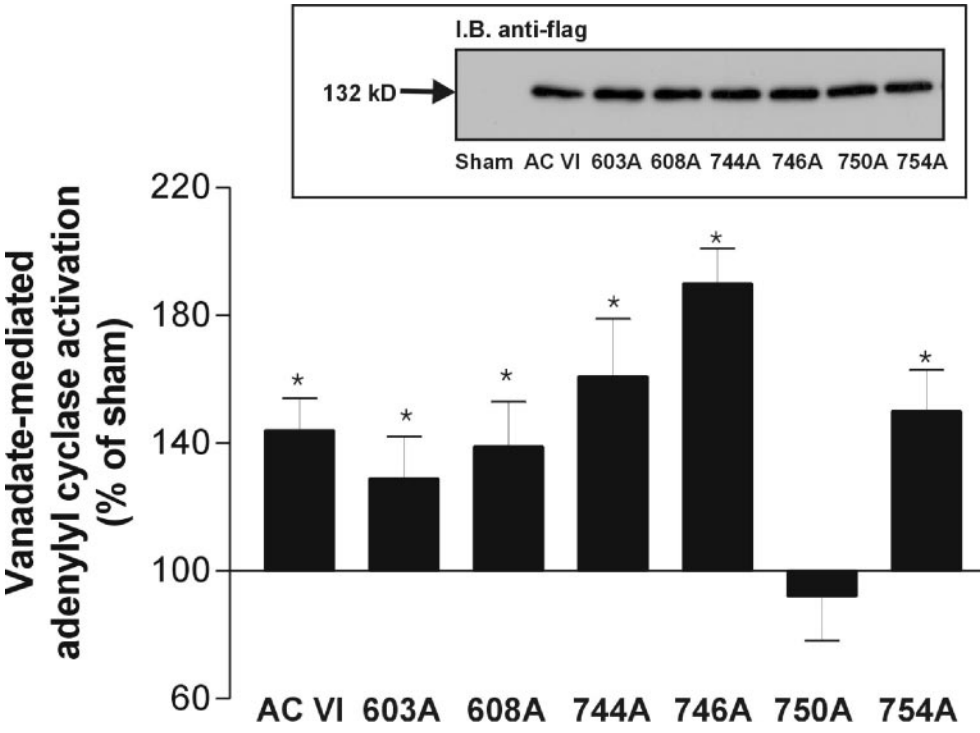

Fig. 3. Enhancement of vanadate-mediated adenylyl cyclase activation in AC VI-transfected HEK 293 cells: effect of single serine > alanine mutagenesis. Adenylyl cyclase activity was assessed in sham-, AC VI-, or single mutant-transfected cells after vanadate (300 μM) treatment. The data are expressed as the percentage of increase of vanadate-stimulated adenylyl cyclase activity versus sham control cells. [(Vandate (+ forskolin)-stimulated activity in AC_x/forskolin-stimulated activity in Ac_x) ÷ (vandate (+ forskolin)-stimulated activity in sham/forskolin-stimulated activity in sham)] ∗, $p < 0.05$ versus sham-transfected cells. Inset depicts expression of AC VI and single mutants from whole cell lysates.

demonstrated between AC VI and both c-Raf and b-Raf, 3) that Ser750 and both Ser603 and Ser608 are the critical residues underlying the Raf-mediated phosphorylation/sensitization of adenylyl cyclase VI (and that Ser750 is critical for catalytic activity of adenylyl cyclase beyond its role in c-Raf-mediated regulation), and 4) that the effects of Raf kinase on adenylyl cyclases are isoform-selective.

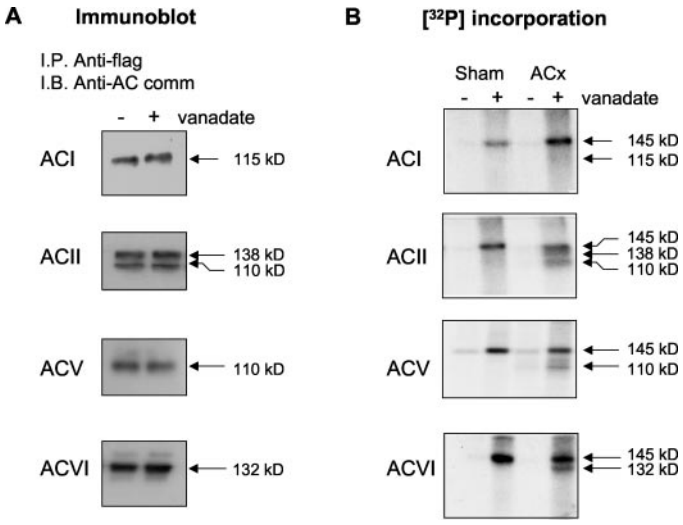

Fig. 4. The pattern of tyrosine kinase-mediated phosphorylation of adenylyl cyclases parallels the pattern of tyrosine kinase-mediated sensitization of adenylyl cyclase. Expression of adenylyl cyclase isoforms was assessed by Western blotting (A). HEK 293 cells were transiently transfected with empty vector (sham) or vectors containing adenylyl cyclase isoforms I, II, V, or VI cDNA. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, immune complexes were resolved and immunoblotted with anti-AC comm antibody. Effect of vanadate (300 μM) treatment on phosphorylation state of adenylyl cyclase isoforms (B). HEK 293 cells transiently transfected with FLAG-tagged adenylyl cyclase isoforms and labeled with [γ-^{32}P]ATP. Cell lysates were then subjected to immunoprecipitation with anti-FLAG antibody and resolved on SDS-PAGE. Data are representative of three separate experiments.

Our initial report had suggested a role of Raf kinase in the tyrosine kinase-mediated serine phosphorylation of adenylyl cyclase (Tan et al., 2001). The basis of our evidence, however, was indirect (i.e., using relatively nonspecific serine-threonine kinase inhibitors and a single dominant-negative mutant of Raf kinase). In addition, we did not determine whether there might be a direct interaction (i.e., whether adenylyl cyclase isoform VI could associate with Raf kinase). The current study demonstrates that in a reconstituted system, adenylyl cyclase isoform VI can still be phosphorylated by a c-Raf-dependent mechanism. Furthermore, our transfection study demonstrates a physical association between these enzymes that seems to be constitutive (and not dependent on the pre-existing extent of activation of either enzyme). At the time of our initial report it was believed that MEKK was the sole substrate for c-Raf kinase. It is notable that subsequent to the publication of our initial report, several other substrates of c-Raf kinase have been identified including CK2, nuclear factor-κB, and ASK1 (for review, see Hindley and Kolch, 2002). The current study suggests yet another candidate target for Raf kinase; however, it should be emphasized that because the source of adenylyl cyclase VI is an enriched (but not purified) membrane preparation (Pieroni et al., 1995) that the requirement for an additional component in the Raf kinase-adenylyl cyclase VI interaction still cannot be definitively ruled out. Our data do suggest, however, that, even if there is an intermediate serine kinase involved in this mechanism, it must be closely associated with the adenylyl cyclase/Raf kinase complex.

Regarding the physical association of adenylyl cyclase and Raf kinase, it is notable that prior studies had identified the existence of other scaffolding complexes linking tyrosine kinase and G-protein-coupled receptor-linked systems—primarily those at the level of G-protein-coupled receptor/β-arrestin including both extracellular signal-regulated kinase, Src kinase, and epidermal growth factor receptor (Pierce et

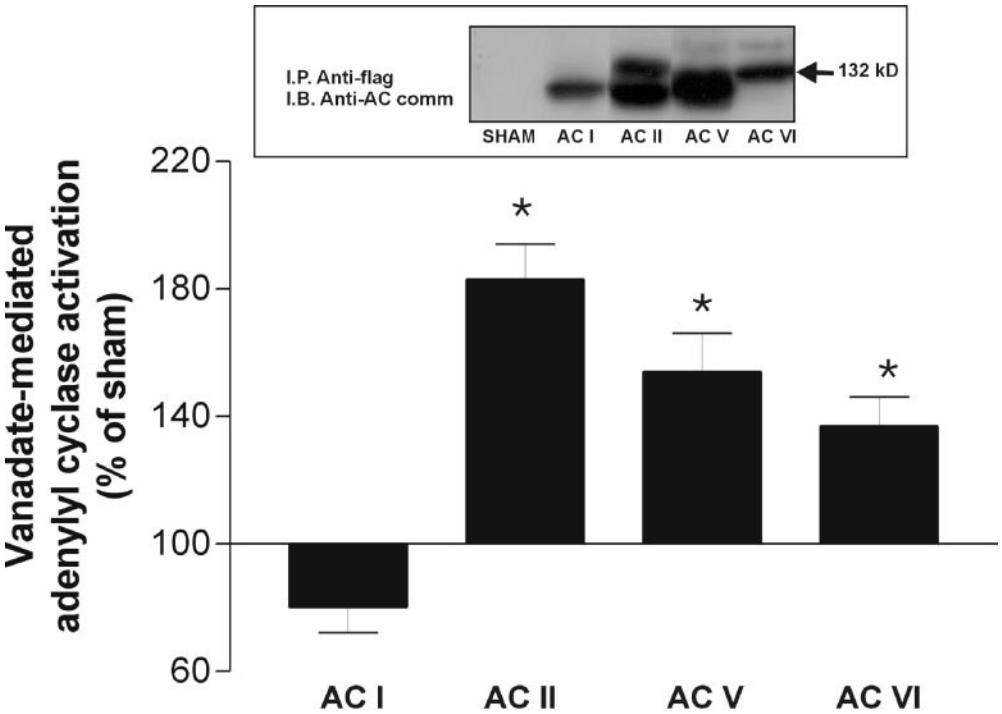

Fig. 5. Effect of vanadate on adenylyl cyclase isoform activation. Adenylyl cyclase activity was measured 72 h after transfection in the absence or the presence of sodium vanadate (300 μM). The data are expressed as the percentage of increase in vanadate-stimulated adenylyl cyclase activity versus sham control cells. [(Vandate (+ forskolin)-stimulated activity in AC_x/forskolin-stimulated activity in AC_x) ÷ (vandate (+ forskolin)-stimulated activity in sham/forskolin-stimulated activity in sham)] *, $p < 0.05$ versus sham-transfected cells. Inset depicts expression of various AC isoforms. Cell lysates, obtained from cells used in adenylyl cyclase activity experiments, were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-adenylyl cyclase antibody (AC comm).

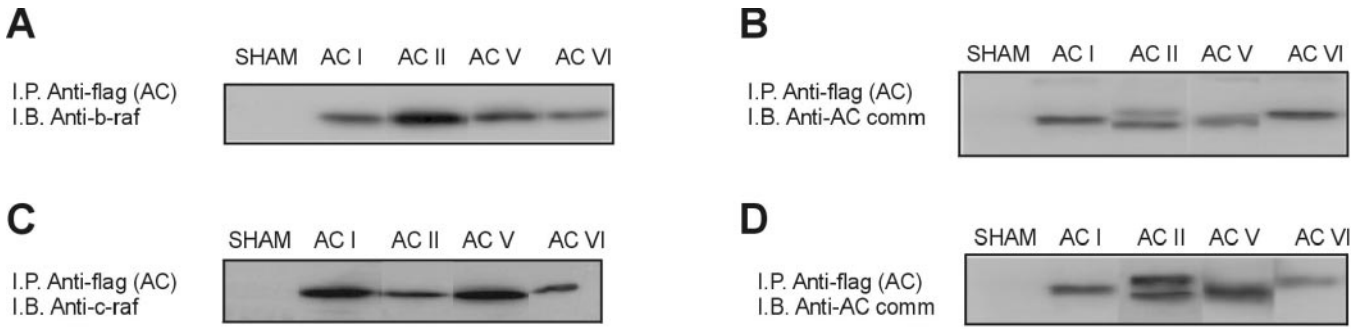

Fig. 6. Association of adenylyl cyclase isoforms with either b-Raf kinase (A) or c-Raf kinase (C). HEK 293 cells were cotransfected with FLAG-tagged adenylyl cyclase isoforms (or vector for sham cells) and either b-Raf kinase or c-Raf kinase cDNA. The presence of b-Raf kinase or c-Raf kinase in anti-FLAG immune complexes was detected by Western blotting. The blots depicted are representative of three experiments performed under identical conditions. B and D depict the adenylyl cyclase isoforms after anti-FLAG immunoprecipitation and immunoblotting with AC comm antibody.

TABLE 3

Forskolin- and vanadate-stimulated adenylyl cyclase activity in HEK-transfected cells

Adenylyl cyclase activity was expressed as picomoles per minute per milligrams of protein. Vanadate-stimulated activity was expressed as the increase in activity above forskolin-stimulated activity. The data represent the mean ± S.E.M. from five to seven separate experiments.

Transfected Plasmid	Forskolin-Stimulated Activity	Vanadate-Stimulated Activity
	pmol/min/mg	
Sham	715 ± 43	587 ± 95
AC I	978 ± 80*	743 ± 53
AC II	1534 ± 142*	2744 ± 380*
AC V	2490 ± 220*	3617 ± 256*
AC VI	924 ± 51*	1041 ± 78*

* $p < 0.05$ versus sham-transfected cells.

al., 2001). The present study demonstrates but another point of interaction in what now must be seen as highly cross-regulated pathways.

In our previous studies, we demonstrated that serine to alanine mutagenesis of residues in either the C1b region of the enzyme (S603/S608) or in the IC4 region of the molecule (S744/S746/S750/S754) impaired tyrosine kinase-mediated phosphorylation and sensitization of adenylyl cyclase (Tan et al., 2001). The current study identifies that in the IC4 region a single serine to alanine mutation recapitulated the phenotype of the previously characterized "block mutations" of serine to alanine at amino residues 744, 746, 750, and 754. It is notable, however, that the single serine to alanine mutation at amino acid residue 750 resulted in the expression of an enzyme that was unresponsive both to tyrosine kinase-mediated sensitization (with vanadate) and to direct activation with forskolin. This suggests that the serine 750 is critical both for tyrosine kinase-mediated regulation as well as more generally for enzyme function. In contrast, serine to alanine mutations at both amino acid residues 603 and 608 seem to be necessary to impair the tyrosine kinase-mediated phosphorylation and sensitization of adenylyl cyclase (Tan et al., 2001) because mutagenesis of either single residue did not impair the extent of Raf kinase-mediated phosphorylation/sensitization of AC VI. In fact, the single serine to alanine mutation at amino acid residue 608 resulted in enhanced forskolin and vanadate-mediated adenylyl cyclase activation in HEK 293 cells. The C1b region of adenylyl cyclase VI is important in the regulation of adenylyl cyclase function and is the site of both protein kinase A- and protein kinase C-mediated phosphorylation (Premont et al., 1992; Chen et al., 1997; Lai et al., 1997; Lin et al., 2002). Whether the single serine to alanine mutation at 608 might enhance forskolin binding, relieve inhibitory regulatory mechanisms, or alter the binding of an additional regulator of AC activity remains to be determined.

The effects of other kinases (including protein kinase A and protein kinase C) on adenylyl cyclase have been shown to be isoform-specific (Yoshimura and Cooper, 1993). The present study suggests that the effects of Raf kinase are similarly isoform-selective. It is interesting that, although all isoforms tested could be shown to associate with c-Raf and b-Raf kinase, this association did not universally result in phosphorylation/activation in all AC isoforms. Furthermore, the ability to phosphorylate adenylyl cyclase is selective for Raf kinases. In aggregate, these data are consistent with the hypothesis that the physical association of these enzymes may be required but is clearly not sufficient for the Raf kinase-mediated phosphorylation/ sensitization of adenylyl cyclases. This would suggest that the sites for Raf "binding" and Raf-mediated phosphorylation occur at discrete regions of the adenylyl cyclases. The sites for Raf-mediated phosphorylation of adenylyl cyclases are unlikely to be identical between the isoforms. It is noteworthy that there is little sequence homology at either amino acids 603 and 608 or amino acid 750 in the isoforms examined. Thus, the common effects of Raf kinase to enhance adenylyl cyclase activity and phosphorylation of isoforms II, V, and VI must involve different sites/domains in each isoform.

In summary, this study demonstrates a direct physical association of Raf kinases and adenylyl cyclase, which seems to be generalized across the subfamilies of adenylyl cyclase isoforms. The functional consequences of these interactions, however, are isoform-selective (both in terms of specific adenylyl cyclase isoforms as well as Raf kinase isoforms). Overall, these functional interactions between adenylyl cyclases and Raf kinases suggest an important but previously unrecognized protein-protein interaction between these two key regulatory enzymes.

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