Tyrosine Kinase-Mediated Serine Phosphorylation of Adenylyl Cyclase†

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ABSTRACT: Receptor tyrosine kinase (RTK) activation is associated with modulation of heptahelical receptor-stimulated adenylyl cyclase responses. The mechanisms underlying the RTK-mediated enhancement of adenylyl cyclase function remain unclear. In the present studies, we show that the tyrosine kinase-dependent enhancement of adenylyl cyclase isoform VI function parallels an enhancement in serine phosphorylation of the enzyme. This effect was mediated by both RTK activation, with IGF-1, and by tyrosine phosphatase inhibition, with sodium orthovanadate. This enhancement of adenylyl cyclase function was not attenuated by inhibitors of ERK, PKC, PKA, or PI3 kinase activity but was blunted by inhibition of endogenous p74^{raf-1} activity. To characterize the molecular site of this effect we identified multiple candidate serine residues in and adjacent to the adenylyl cyclase VI C1b catalytic region and performed serine-to-alanine site-directed mutagenesis using adenylyl cyclase VI as a template. Mutation of serine residues 603 and 608 or serine residues 744, 746, 750, and 754 attenuated both the tyrosine kinase-mediated enhancement of enzyme phosphorylation as well as the sensitization of function. Together, these data define a novel tyrosine kinase-mediated mechanism leading to serine phosphorylation of adenylyl cyclase isoform VI and the sensitization of adenylyl cyclase responsiveness.

Intracellular adenosine 3′, 5′-monophosphate (cyclic AMP)[1] concentrations are primarily regulated by alterations in its rate of synthesis. The activity of the enzyme adenylyl cyclase (AC), which catalyzes the generation of cyclic AMP from adenosine triphosphate (ATP), is itself regulated by both extracellular stimuli (via GPCR activation) and intracellular stimuli. Regulation of adenylyl cyclase activation is primarily via G−protein interactions or calcium/calmodulin interactions (*1*). Adenylyl cyclase catalytic function is also regulated directly. Previous studies have demonstrated that adenylyl cyclase activation is regulated by varying ion concentrations (e.g., magnesium and calcium, ref *1*). Additionally, covalent modification of the enzyme by phosphorylation has been described which occurs dynamically and alters function (*2*−*8*).

Serine/threonine phosphorylation of specific isoforms of adenylyl cyclase have been described, mediated both by protein kinase A (PKA) and protein kinase C (PKC). PKC-mediated phosphorylation of adenylyl cyclase enhances catalytic function of isoforms 1, 2, 3, and 5 (*2*−*5*) whereas PKC-mediated phosphorylation of isoform 6 is inhibitory (*6*). PKA-mediated phosphorylation of isoform 6 is inhibitory (*7*, *8*).

More recently, it has been appreciated that adenylyl cyclase activity is regulated by the activation of both receptor- and nonreceptor-linked tyrosine kinases (*9*−*13*). Notably, a range of effects of both receptor and nonreceptor tyrosine kinases has been demonstrated at multiple levels of GPCR signaling pathways including at the level of the receptor [both stimulatory (*14*) and inhibitory (*15*)] and G−proteins (*16*) and downstream at the level of cAMP-dependent phosphodiesterase (*17*, *18*) (where the effect of insulin to activate phosphodiesterase activity parallels that of GPCR-mediated phosphodiesterase activation). Generally, the effect of tyrosine kinase activation 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 both receptor and heterotrimeric G protein activity that were detectable at the level of PKA activation (even under conditions where phosphodiesterase activity was **not** inhibited) (*19*−*21*). However, the molecular mechanism(s) underlying the direct sensitization of adenylyl cyclase catalytic activity remained

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[1] Abbreviations: cAMP, adenosine 3′,5′ -monophosphate; GPCR, G protein-coupled receptor; RTK, receptor tyrosine kinase; IGF-1, insulin-like growth factor-I; ERK, extracellular regulated kinase; PKA, protein kinase A; PKC, protein kinase C; PI 3 kinase, phosphatidylinositol 3-kinase; AC6; adenylyl cyclase isoform 6; NΔraf, dominant negative inhibitor of raf-1 kinase; p74^{raf-1}, raf-1 kinase.

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Table 1: Oligonucleotides Employed to Generate Site-Specific Serine to Alanine FAC6 Mutants[a]

candidate senine residues for mutation	primer name	primer orientation	primer sequence
588	S588A_fp	5′−3′	CGGGCCAACGCTATGGAAGGAC
	S588A_rp	3′−5′	GTCCTTCCATAGCGTTGGCCCG
603, 608	S6038A_fp	5′−3′	CGTGCCTTCGCACGGACCAAGGACGCGAAGGCATTCC
	S6038A_rp3	3′−5′	CTTGGCGTCCTTGGTCCGAGCGAAG
674	S674A_fp	5′−3′	GAGGATCTCGAGAAGAAGTATGCCCGGAAAG
	S674A_rp	3′−5′	CTTTCCGGGCATACTTCTTCTCGAGATCCTC
744, 746,750, 754	S7454A_fp	5′−3′	GCGCCTGGCACGCGCTATCGTCCGCGCACGGGTGC-ACGCAACGGCTGTTGG
	S7454A_rp	3′−5′	GACTGGCCGTTGCGTGCACCCGTGCGCGGACGATA-GCGCGTGCCAGGCGC
restriction enzyme sites			
*Nhe*I	NheI_fp	5′−3′	CACCG**GCTAGC**GCAG
*Bsu*36I	Bsu36I_fp	5′−3′	CTGAAC**CCTGAGG**ATGAGG
	Bsu36I_rp	3′−5′	CTTCAT**CCTCAGG**GTTCAG
*Eco*47III	Eco47III_rp	3′−5′	CATGTATTTG**AGCGCT**ACCCTCCC
		round 1	round 2
cassette	PCR 1	PCR 2	
S588A	NheI_fp, S588A_rp	S588A_fp, Bsu36I_rp	NheI_fp, Bsu36I_rp
S6038A	NheI_fp, S6038A_rp3	S6038A_fp, Bsu36I_rp	NheI_fp, Bsu36I_rp
S7454A	Bsu36I_fp, S7454A_rp	S7454A_fp, Eco47III_rp	Bsu36I_fp, Eco47III_rp

[a] Three micrograms of wild-type Flag AC6 was digested using the restriction enzymes *Nhe*I + *Bsu*36I, or *Bsu*36I + *Eco*47III. Digests were resolved via agarose gel electrophoresis and the linearized *Nhe*I-, *Bsu*36I-, and *Bsu*36I-, *Eco*47III-digested wild-type Flag AC6 was gel purified from their respective cassettes. The mutated AC6 cassettes harboring the various S→A mutation(s) were correspondingly digested and ligated into the wild-type Flag AC6.

unknown. In the present study, we demonstrate tyrosine kinase-dependent sensitization of adenylyl cyclase activity involves raf-1 kinase ($p74^{raf-1}$)-mediated serine phosphorylation of the catalytic domain of adenylyl cyclase isoform 6 (AC6). In doing so, we define a novel phosphorylation-dependent mechanism leading to enhanced adenylyl cyclase-mediated increases in intracellular cAMP levels.

MATERIALS AND METHODS

Epitope Tagging and Mutagenesis. The cDNA pcDNAI-AC6 encoding rat AC6 was kindly provided by Drs. R. Iyengar and J. Pieroni, Mount Sinai School of Medicine, City University of New York, New York. The 24 base pair flag epitope GACTACAAGGACGACGATGACAAG was incorporated at the 5′ amino terminal of AC6 via PCR mutagenesis using the 53-mer forward primer ACF2 (TCAAGCTTATGGACTACAAGGACGACGATGACAAGCCCCTGCCCGTGGCCCGA; which contains a *Hin*dIII restriction site, an initiator methionine, the flag epitope, and amino acids 2−7 of AC6), and the 29-mer reverse primer ACR3555 (TCTCTAGACTAACTGCTGGGGCCCCCATT; which contains an *Xba*I restriction site followed by a stop codon). The PCR product was directly cloned into the TA cloning vector pCR2.1 (Invitrogen, San Diego, CA). Flag epitope-tagged AC6 (flag AC6) was excised as a *Hin*dIII−*Xba*I 3.6 kilobase fragment and subcloned into the 5.5 kilobase mammalian expression vector pRc/CMV (Invitrogen, San Diego, CA) to yield pRc/CMV−flag AC6. pRc/CMV−flag AC6 was verified via restriction enzyme digestion for size and orientation. Positive recombinants were verified by sequencing and subsequently purified via two sequential rounds of cesium chloride/ethidium bromide equilibrium centrifugation.

Eight serine residues (serines 588, 603, 608, 744, 746, 750, and 754) localized within the catalytic C_{1b} region and in the presence of contiguous basic amino acid motifs [either amino- or carboxyl-terminal to the serine residue(s)] were identified and subjected to PCR-based site-directed mutagenesis. Three serine (S) to alanine (A) flag AC6 mutants were generated: (1) S588A, (2) S744A, S746A, S750A, and S754A (mutant A), and (3) S603A and S608A (mutant B). Mutants were generated via overlap-extension PCR mutagenesis (*22*) using flag AC6 as the template. Briefly, two independent PCR reactions were carried out to generate a pair of overlapping PCR products. The first reaction used an upstream, sense mutagenizing oligonucleotide paired with a downstream, antisense oligonucleotide harboring a unique restriction enzyme digestion site. The second PCR reaction used an upstream sense oligonucleotide paired with a downstream, antisense mutagenizing oligonucleotide harboring a unique restriction enzyme digestion site (see Table 1). Each PCR product was resolved by agarose gel electrophoresis, doubly purified and concentrated using the GeneClean II kit (Bio101, Bio/Can Scientific, Mississauga, ON) followed by the Qiaquick PCR purification method (Qiagen, Mississauga, ON). Equivalent amounts (∼50 ng) of each purified, PCR product from the first paired reactions were combined and amplified using the extreme sense upstream and antisense downstream primers. The resulting PCR product was purified as above and sequenced to ensure the serine residue(s) of interest were mutated to alanine residue(s). The verified, mutated PCR product was double digested with the terminal restriction enzymes, and this mutant cassette was ligated into wild-type flag AC6, which had been digested with the identical restriction enzymes to eliminate the corresponding wild-type cassette. Transformants were screened by restriction enzyme digestion analysis for size and orientation, and positive insertion clones were resequenced to verify that (1) the open reading frame was intact and error free and (2) to ensure that each serine to alanine mutation was maintained. Verified mutants were

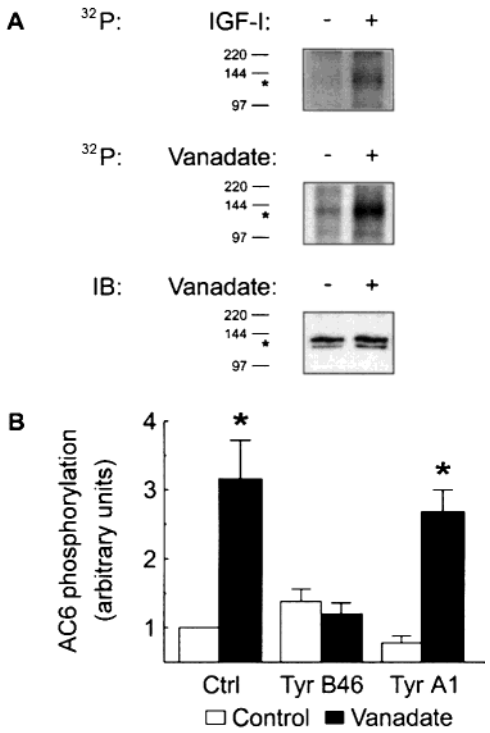

FIGURE 1: Effect of tyrosine kinase activation on adenylyl cyclase phosphorylation. (A) HEK 293−flag AC6 cells were labeled with 0.5 mCi/mL [^{32}P]orthophosphate and treated in the absence (−) or presence (+) of 100 nM IGF-I (^{32}P; upper panel) or 300 μM vanadate (^{32}P; middle panel). Cells were lysed, flag AC6 immunoprecipitated and resolved as indicated in Methods and Materials. Unlabeled cells were handled identically except were subjected to immunoblotting to determine total amounts of flag AC6 protein (IB; lower panel). Autoradiographs are representative of the results from at least five experiments conducted under identical conditions. Asterisk denotes flag AC6. (B) Densitometric analysis of the tyrosine kinase-dependent flag AC6 phosphorylation. HEK 293−flag AC6 cells were pretreated with or without 100 μM tyrphostin B46 (Tyr B46) or 100 μM tyrphostin A1 (Tyr A1) in the absence (control) or presence of 300 μM vanadate and assessed for adenylyl cyclase phosphorylation. Data represents the mean ± SEM from 12 experiments performed under identical conditions. In each experiment, the extent of flag AC6 phosphorylation in the absence of vanadate and inhibitors was assigned a value of 1.

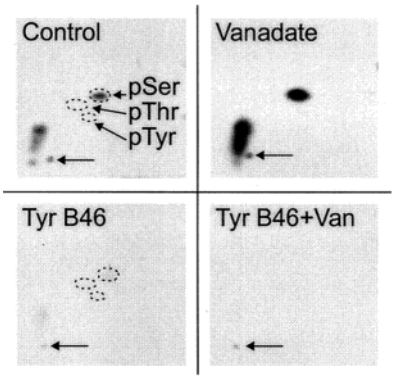

FIGURE 2: Effect of tyrosine kinase activation on adenylyl cyclase. [^{32}P]orthophosphate-labeled HEK 293−flag AC6 cells were pretreated with or without 100 μM tyrphostin B46 (Tyr B46) in the absence (control) or presence of 300 μM vanadate. Cells were lysed, flag AC6 immunoprecipitated and resolved as indicated in the Methods and Materials. Flag AC6 immunoprecipitates derived from these phosphorylation experiments were then rehydrated and precipitated from the dried gel and subjected to partial amino acid hydrolysis. Hydrolysates were lyophilized and resuspended in pH 1.9 running buffer with cold phosphoamino acid standards. Hydrolysates were spotted on thin layer cellulose plates and subjected to two-dimensional separation plates and autoradiography. Arrow indicates application origin. Autoradiographs are representative of the results from three experiments conducted under identical conditions.

purified using the Endotoxin-free Maxiprep Kit (Qiagen) to obtain transfection-quality cDNA.

Cell Culture and Transfections. HEK 293 cells were cultured in Dulbeccos's Modified Eagle's Medium (D-MEM) with 5% fetal calf serum as previously described (*20*). Transient transfection using 10 μg of either sham (pRc/CMV vector backbone), 10 μg of flag AC6 or 10 μg of flag AC6 mutants was performed using a modified calcium phosphate method (*23*). In studies where NΔraf (10 μg) or MEK inhibitor (10 μg) was coexpressed with flag AC6, the total amount of DNA transfected was kept constant by using appropriate amounts of empty vector.

Assessment of Adenylyl Cyclase Activity. Assays of adenylyl cyclase activity were performed as previously described (*21*) but were modified to accommodate for a cell suspension preparation, where 20−40 μg permeabilized cells were incubated a final assay volume of 100 μL. In studies conducted with kinase inhibitors, cells were permeabilized and assayed in the presence of the respective inhibitor or vehicle control. All inhibitors were resuspended as per the manufacturer's instructions (all inhibitors obtained from Calbiochem-Nova biochem, La Jolla, CA), where the final diluent was less than 0.1%, a concentration which has no effects on adenylyl cyclase activity (data not shown).

Western Blotting. At 72 h posttransfection, confluent cells were rinsed in phosphate-buffered saline (PBS) and lysed in 1× lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris, pH 8.0, 2 mM EDTA, 100 μM phenylmethanesulfonyl fluoride, 1 μg/mL each of antipain, chymostatin, leupeptin, and pepstatin A). Control and vanadate-treated HEK 293−flag AC6 cells were then scraped with a rubber policeman and further homogenized using ice-cold Potter Elvehjem hand homogenizers (VWR, Mississauga, ON). Lysates were then transferred to ice-cold microcentrifuge tubes and clarified by centrifugation for 5 min at 12000*g* at 4 °C. The cleared lysates were incubated with anti-flag M2 antibody conjugated to agarose beads (Sigma, St. Louis, MO) overnight at 4 °C on a rocker platform. The samples were then centrifuged for 5 min at 12000*g* at 4 °C. The resulting pellet was washed four times with ice-cold lysis buffer and resuspended in sample buffer (125 mmol/L Tris, pH 6.8, 100 mmol/L dithiothreitol, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, and 0.025% bromophenol blue). Samples were incubated at 65 °C for 15 min and centrifuged for 5 min at 12000*g* at 4 °C to pellet agarose beads. Protein molecular weights were dissolved in the same buffer. Supernatants resolved via SDS−PAGE as described previously (*24*). Immunoblotting was performed by incubating the membranes with anti-AC5/6 (Santa Cruz Biotechnology, Santa Cruz, CA).

Phosphorylation Experiments. Two methods ([^{32}P]orthophosphate or [γ^{32}P]ATP) were employed for phosphorylation studies. At 72 h post transfection, confluent HEK 293−flag AC6 cells cultured in T25 cm^2 flasks were washed with phosphate-free MEM (pH 7.4; Life Technologies Gibco BRL, Burlington, ON) and labeled with 0.5 mCi/mL [^{32}P]-orthophosphate (Amersham, Oakville, ON) with or without

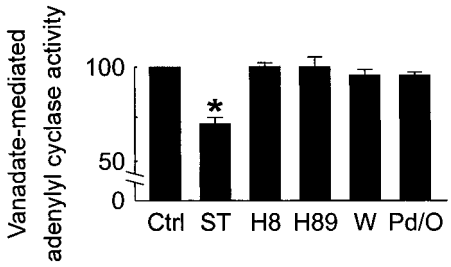

FIGURE 3: Effect of serine/threonine kinase inhibitors on the vanadate-mediated enhancement of adenylyl cyclase activity. HEK 293−flag AC6 cells were treated with or without the respective inhibitors 1 μM staurosporine (ST), 100 μM H8, 600 nM H89, 100 nM wortmannin (W), or 100 μM PD9059 + 100 μM olomoucine (Pd/O) in the absence or presence of 300 μM vanadate and were assessed for forskolin-stimulated adenylyl cyclase activity. For each experiment, the vanadate-mediated increases in forskolin-stimulated adenylyl cyclase activity in the absence of all kinase inhibitors (control) was assigned a value of 100%. The vanadate-mediated increases in adenylyl cyclase activity in the presence of the respective inhibitors were normalized to control. Average forskolin-stimulated activity: 79.5 pmol/min/mg of protein; average forskolin-stimulated activity + 300 μM vanadate; 178.9 pmol/min/mg of protein. Data represents the mean ± SEM from five experiments performed under identical conditions.

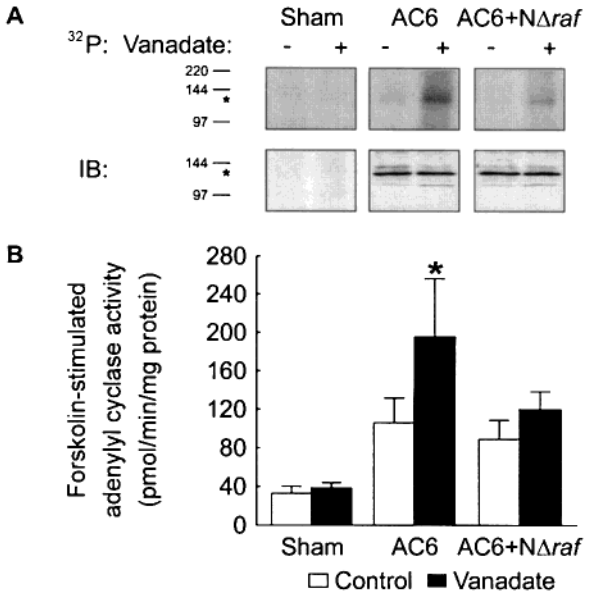

FIGURE 4: Effect of inhibition of raf-1 activity on vanadate-mediated phosphorylation of adenylyl cyclase and enhancement of adenylyl cyclase activity. (A) Sham cells, HEK 293−flag AC6 cells, or HEK 293−flag AC6 cells transfected with the dominant negative raf-1 inhibitor NΔraf were labeled with 0.5 mCi/mL [^{32}P]orthophosphate and treated in the absence (−) or presence (+) of 300 μM vanadate (^{32}P; upper panel). Cells were lysed, flag AC6 immunoprecipitated, and resolved as indicated in the Methods and Materials. Unlabeled cells were handled identically except were subjected to immunoblotting to determine total amounts of flag AC6 protein (IB; lower panel). Asterisk denotes flag AC6. The autoradiographs depicted are representative of the results from three experiments conducted under identical conditions. (B) Adenylyl cyclase activity in sham cells, HEK 293−flag AC6 cells, or HEK 293−flag AC6 cells transfected with NΔraf. Cells were treated in the absence (control) or presence of 300 μM vanadate and were assessed for forskolin-stimulated adenylyl cyclase activity. Data represents the mean ± SEM from nine experiments performed under identical conditions.

300 μmol/L vanadate in phosphate-free MEM for 4 h at 37 °C in a humidified incubator. Control and vanadate-treated HEK 293−flag AC6 cells were then washed extensively with phosphate-free MEM, lysed, and homogenized as described above. Flag AC6 was immunoprecipitated from clarified supernatants using anti-flag M2 antibody-agarose, washed, and resuspended in sample buffer. Samples were incubated at 65 °C for 15 min, agarose pelleted, and immunocomplexes resolved. Alternatively, cells were permeabilized and resuspended at a final concentration of 1 mg/mL in buffer B (Hanks' Balanced Salt Solution, pH 7.4, 33 mM HEPES, 1.25 mM EDTA, and 5 mM MgSO4). Permeabilized cell suspension (200−300 μg) was incubated in an assay volume of 500 μL of buffer B with 0.1 mCi/mL [γ^{32}P]ATP in the absence or presence of 300 μM vanadate for 30 min at 30 °C on an inversion rocker. Incubations were terminated following the addition of 500 μL of ice-cold 2× lysis buffer in the presence or absence of 100 μM vanadate. Flag AC6 was immunoprecipitated and handled as above. SDS−PAGE was performed using the procedure described by Laemmli (*25*). A 10% resolving gel with a 4% stacking gel was used for all studies (model SE-400 gel apparatus; Hoefer Scientific Instruments, San Francisco, CA). Electrophoresis was performed at a fixed current of 10 mA/gel slab for 15−18 h. Gels were stained in 2 g/L Coomassie brilliant blue R-250 (Bio-Rad, Mississauga, ON), 50% methanol, and 10% acetic acid for 2 h. The gels were mounted in cellophane, air-dried overnight, and exposed to X-ray film. Exposures were analyzed via densitometic scanning using the NIH 6T scanning program (NIH, Bethesda, MD).

Phosphoamino Analysis. Phosphoamino acid analysis by two-dimensional separation on thin layer cellulose plates was carried out according to the method of Boyle (*26*). Following the phosphorylation experiments, the protein bands corresponding to immunoprecipitated flag AC6 were identified by lining up the exposure with the dried gel. Flag AC6 was carefully excised and gel slices were transferred to respective microcentrifuge tubes and 400 μL of freshly prepared 50 mmol/L ammonium bicarbonate (pH 7.3−7.6; BDH, Toronto, ON) was added to rehydrate the gel slices. The cellophane backing was removed using forceps, and slices were homogenized using disposable plastic microcentrifuge pestles (Kontos, Vineland, NJ). Pestles were rinsed with 800 μL of 50 mmol/L ammonium bicarbonate to remove residual gel pieces. To the gel suspensions, 12 μL of 2-mercaptoethanol and 12 μL of 10% SDS (both from Bio-Rad, Mississauga, ON) were each added and tubes capped. After vortexing briefly, slurries were boiled for 5 min and tumbled overnight. The following day, gel suspensions were briefly vortexed followed by centrifugation to compress gel bits. The supernatants were transferred to fresh microcentrifuge tubes and 200 μL of 50 mmol/L ammonium bicarbonate was added to the slurries and tumbled for 2 h to wash the gel bits and elute any residual protein. After the second elution step, the gel bits were pelleted by centrifugation, and the second supernatant was combined with the first. The pooled supernatants were clarified via centrifugation for 10 min at 12000*g* and transferred to fresh microcentrifuge tubes and 20 μg of γ-Globulin (1 mg/mL; Sigma-Aldrich Canada Ltd., Oakville, ON) and 200 μL of ice-cold trichloracetic acid (100% TCA; BDH, Toronto, ON) was added. Samples were mixed thoroughly and placed on ice for 60 min. TCA precipitates were centrifuged for 10 min at 12000*g* at 4 °C, supernatants carefully discarded, and precipitates washed

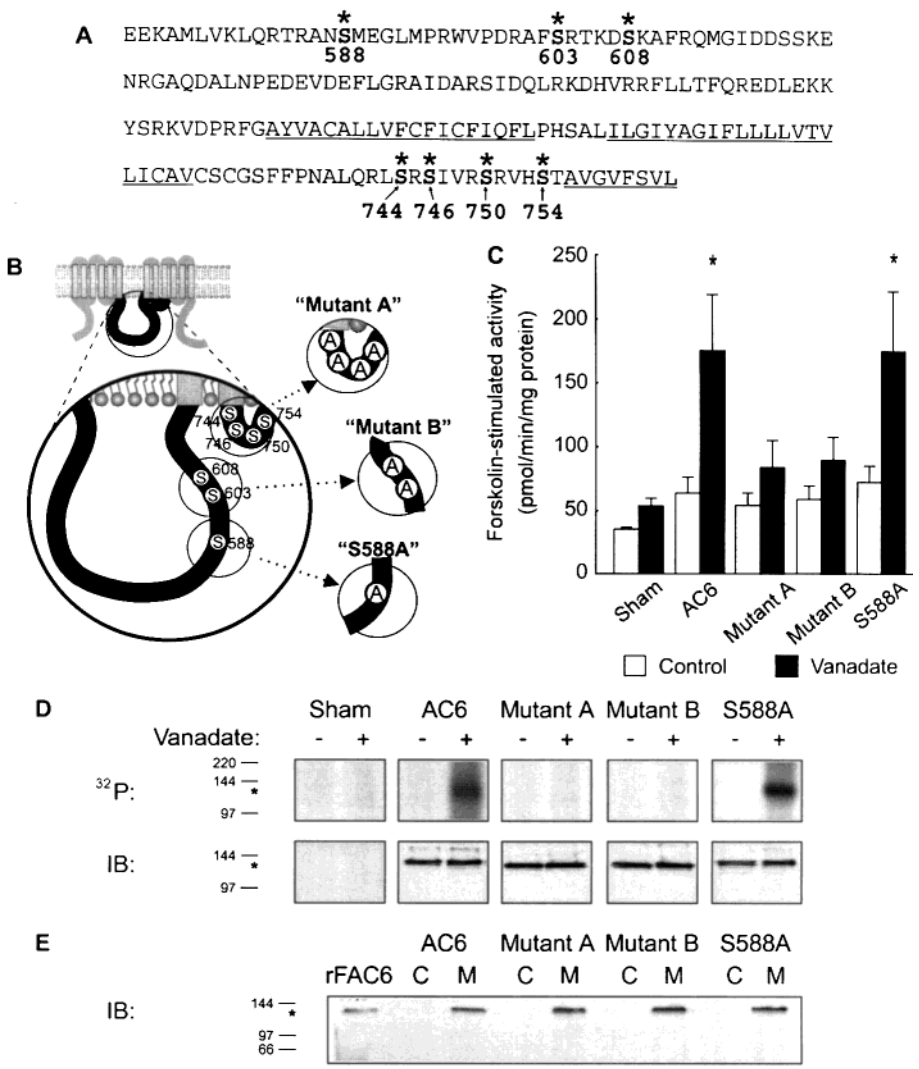

FIGURE 5: Primary AC6 amino acid sequence analysis. (A) Residues 514−763 depicting candidate C_{1b} and IC4 serine residues (asterisk) and transmembrane domains (underlined). (B) Schematic two-dimensional representation of AC6 serine residue localization and corresponding individual flag AC6 mutants (mutant A, mutant B, S588A). (C) Adenylyl cyclase activity in sham cells, HEK 293−flag AC6, or HEK 293−flag AC6 mutant cells. Cells were treated in the absence (control) or presence of 300 μM vanadate and were assessed for forskolin-stimulated adenylyl cyclase activity. Data represents the mean ± SEM from six experiments performed under identical conditions. (D) Effect of vanadate on adenylyl cyclase phosphorylation. Permeabilized sham cells, HEK 293−flag AC6 cells, or HEK 293−flag AC6 mutant cells were labeled with 0.1 mCi/mL [γ^{32}P]ATP and treated in the absence (−) or presence (+) of 300 μM vanadate (^{32}P; upper panel). Cells were lysed, flag AC6 immunoprecipitated and resolved as indicated in the Methods and Materials. Unlabeled cells were handled identically except were subjected to immunoblotting to determine total amounts of flag AC6 protein (IB; lower panel). Autoradiographs are representative of the results from three experiments conducted under identical conditions. Asterisk denotes flag AC6. (E) Cellular distribution of adenylyl cyclase. HEK 293−flag AC6 cells or HEK 293−flag AC6 mutant cells were lysed, flag protein immunoprecipitated and resolved as indicated in the Methods and Materials. Equivalent amounts of membrane-bound (M) flag AC6 and flag AC6 mutant protein migrate identically with the recombinant flag AC6 protein standard (rFAC6). No adenylyl cyclase was identified in the cytosolic (C) fractions. Asterisk denotes flag AC6 and flag AC6 mutants. Autoradiographs are representative of the results from three experiments conducted under identical conditions.

twice with ice-cold absolute ethanol and allowed to air-dry. Pellets were then subjected to partial amino acid hydrolysis by resuspending the dried samples in 50 μL constant boiling 5.7 mol/L HCl for 60 min at 110 °C. Following hydrolysis, samples were lyophilized in a SpeedVac (VirTis Bench Top 3; The VirTis Company, Gardiner, NY) using KOH traps to collect the acid. Hydrolysates were resuspended in 2−6 μL of fresh buffer, pH 1.9 (7.8% glacial acetic acid, 2.5% formic acid; both from Caledon, Georgetown, ON), which contains 15 parts buffer to 1 part cold phosphoamino acid standards [1.0 mg/mL of each phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) in deionized water (all from Sigma-Aldrich Canada Ltd., Oakville, ON)]. Hydrolysates from control and vanadate-treated HEK 293 cells were spotted (0.25 μL/application) on 100 μm 20 cm × 20 cm cellulose precoated plates (4 samples/plate) without fluores-

Table 2: Adenylyl Cyclase Activity in Wild-Type and AC6 Mutants[a]

	forskolin (100 μM)	Gpp(NH)p (100 μM)	PGE_1 (10 μM) + GppNHp
wild-type	177 ± 51	115 ± 20	224 ± 44
mutant A	156 ± 64	93 ± 13	213 ± 41
mutant B	144 ± 57	91 ± 16	204 ± 31
S558A	144 ± 61	87 ± 14	196 ± 40

[a] Data represent adenylyl cyclase activity (pmol/min/mg of protein) and are the mean ± sem of triplicate measurements from three experiments performed under identical conditions.

Table 3: Regulation of Adenylyl Cyclase Isoforms: Role of Phosphorylation[a]

isoform	kinase	effect	site	references
I	CamK IV	↓↑	S545, S552	Wayman et al., 1996 (*30*)
	$?_{(PKC;\ PE)}$			Choi et al., 1993 (*4*)
II	PKC	↑↑↑	S871, T1057	Bol et al., 1997 (*42*)
	$?_{(PKC;\ PE)}$		T1057	Bol et al., 1997 (*5*)
				Jacobowitz et al., 1993 (*43*), 1994 (*2*), Zimmerman et al., 1996 (*44*), Moughal et al.,1995 (*45*), Tsu et al., 1996 (*46*), Yoshimura et al., 1993 (*47*), Chakrabarti et al., 1998 (*48*)
III	CamK II	↓	S1076	Wei et al., 1996 (*49*), 1998 (*50*)
	$?_{(PKC;\ PE)}$	↑⇔		Choi et al., 1993 (*4*), Yoshimura et al., 1993[b] (*47*)
V	PKA	↓↑		Iwami et al., 1995 (*7*), Premont et al., 1992 (*8*),
	$PKC_{(\zeta)}$			Kawabe et al., 1994 (*3*)
VI	PKA	↓	S674	Chen et al., 1997 (*31*), Premont et al., 1992 (*8*)
	$PKC_{(\zeta\epsilon)}$	↓⇔		Lai et al., 1997 (*6*), Jacobowitz et al., 1994[c] (*2*)
	?(Opioid)	?		Varga et al., 1999 (*51*)
VII	$?_{(PKC;\ PE)}$	↑		Haslauer et al., 1998(52)

[a] Direct adenyly cyclase phosphorylation has not been demonstrated for isoforms IV, VII, VIII, IX. (↑) Enhancement; (↓) inhibition; (⇔) no effect; S, Serine; T, Threonine; ?, unknown; PE, phorbol ester; ζ, PKC isoform zeta; ϵ, PKC isoform epsilon. [b] Alternatively, Yoshimura et al. (1993) demonstrated that phorbol ester had no effect on adenylyl cyclase isoform III activation. [c] Alternatively, Jacobowitz et al. (1994) demonstrated that phorbol ester had no effect on adenylyl cyclase isoform VI activation.

cent indicators (Merck-EM Science, Gibbstown, NJ). Dried plates were prewetted with buffer, pH 1.9, and subjected to electrophoresis in the same buffer using an FBE-3000 Flat Bed water jacketed Electrophoresis Apparatus (Pharmacia, Uppsala, Sweden) precooled to 7 °C at 1000 V for 45 min. Plates were dried using an electric fan and prewetted in buffer, pH 3.5 (5% glacial acetic acid, 0.5% pyridine; Caledon, Georgetown, ON) prior to electrophoresis in the second dimension at 1000 V, 7 °C for 15 min. Following electrophoresis, plates were dried using an electric fan and sprayed with 0.2% 2,2-dihydroxy-1,3-indaneidione (ninhydrin; in acetone; Sigma-Aldrich Canada Ltd., Oakville, ON). Plates were placed in an 80 °C oven (Bio/Can Scientific, Mississauga, ON) for 15 min to visualize cold phosphoamino acid standards and subsequently exposed to X-ray film at −70 °C for 3 days.

Data Analysis. For 2-group comparisons the statistical significance of differences was determined by students *t*-test for unpaired data. $P < 0.05$ on a two-sided test was taken as a minimum level of significance. Dose response curves were analyzed by computerized nonlinear sigmoid curve fitting of the data (Inplot4, GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Initial experiments examined whether growth factor stimulation was associated with increased phosphorylation of AC6 expressed in HEK 293 cells. In response to either IGF-1 (100 nM) or the tyrosine phosphatase inhibitor sodium orthovanadate (300 μM) (*27*−*29*), AC6 phosphorylation was increased (Figure 1A). Vanadate-stimulated AC6 phosphorylation was blocked by the tyrosine kinase inhibitor tyrphostin B46, but was not blocked by the inactive analogue tryphostin A1 (Figure 1B)—consistent with tyrosine kinase-mediated phosphorylation of AC6. While protein phosphorylation constitutes a prominent and dynamic mechanism regulating adenylyl cyclase enzymatic activity (*2*, *6*, *30*), to our knowledge, these data represents the first demonstration of a tyrosine kinase-mediated phosphorylation of an adenylyl cyclase isoform.

To determine whether AC6 was tyrosine phosphorylated in response to vanadate treatment of HEK 293 cells we performed phosphoamino acid analysis. Under baseline conditions, in the absence of vanadate stimulation, basal phosphoserine (pSer), but not phosphotyrosine (pTyr) phosphorylation of AC6 was observed (Figure 2A). This observation is consistent with findings from previous studies (*31*). In response to vanadate stimulation, no increase in pTyr phosphorylation of AC6 was observed. Rather, we observed a robust increase in AC6 pSer content (Figure 2). Treatment of cells with tyrphostin B46 eliminated both basal and vanadate-stimulated pSer phosphorylation of AC6 (Figure 2). The tyrosine kinase-mediated serine phosphorylation-stimulated increases in AC6 enzymatic activity could not be inhibited by PKA (H8), PKC (H89), phosphatidylinositol 3-kinase (PI 3 kinase, wortmannin), or MEK/ERK (PD98059/olomoucine) inhibitors (Figure 3), but were attenuated by the broad-based serine/threonine kinase inhibitor staurosporine. Additionally, the src kinase family inhibitor, PP2 (10 μM), had no significant effect on vanadate-mediated responses (data not shown).

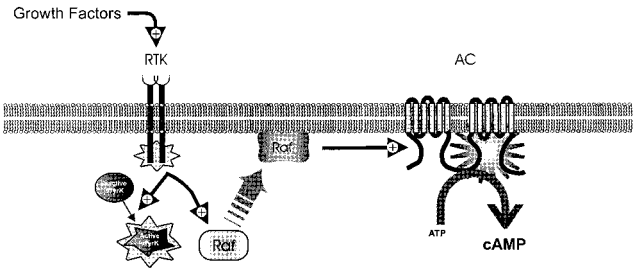

FIGURE 6: Diagrammatic model of tyrosine kinase activation of adenylyl cyclase. Receptor activation initiates signaling cascades recruiting cytosolic tyrosine kinases and tyrosine kinase-activated serine/threonine kinases, including $p74^{raf-1}$, to the membrane. This fosters $p74^{raf-1}$-mediated or $p74^{raf-1}$-activatable kinase serine/threonine phosphorylation of adenylyl cyclase. Serine phosphorylation in a dual C_{1b} and IC4 domain-dependent fashion is mandatory for tyrosine kinase-mediated enhancement of catalytic function (see text).

Many RTK-mediated responses involve the activation of the serine/threonine kinase $p74^{raf-1}$. Further, $p74^{raf-1}$ activity can be inhibited by staurosporine (*32*−*34*). Therefore, we tested whether the coexpression of a dominant-negative $p74^{raf-1}$ inhibitor, NΔraf (*35*, *36*), along with AC6 in HEK 293 cells might attenuate the vanadate-stimulated phospho-

rylation of AC6. The expression of NΔraf had little effect on the basal phosphorylation of AC6, but effectively blocked vanadate-stimulated increases in AC6 phosphorylation (Figure 4A). Consistent with a role for p74^{raf-1} in the functional sensitization of AC6 activity, NΔraf completely abolished the vanadate-mediated enhancement of AC6 activity (Figure 4B). In contrast, coexpression with a dominant negative mutant of MEK (*37*) had no effect on the extent of vanadate-mediated enhancement of forskolin-stimulated adenylyl cyclase activity (AC6 expression alone, 215 ± 20% of forskolin stimulated activity without vanadate; AC6 +MEK DNM, 208 ± 5% $n = 3$).

To further characterize the structural basis of p74^{raf-1}-mediated serine phosphorylation of AC6 and enhancement of adenylyl cyclase activity, we screened for serine/threonine residues within and adjacent to the intracellular C1 catalytic loop that satisfy the broad serine/threonine kinase consensus motif (R/K)−(R/K)−X−S/T−B−(R/K) (*38*). Eight serine amino acid candidate residues within the first catalytic loop subdomain (C_{1b}) and the fourth intracellular loop (IC4) in AC6 fulfilled this criteria (serines 588, 603, 608, 674, 744, 746, 750, and 754) (Figure 5A). We generated three serine (S) to alanine (A) AC6 mutants: (1) S744A, S746A, S750A, S754A (mutant A), (2) S603A, S608A (mutant B), and (3) S588A (Figure 5B). Serine 674 was not evaluated since it was previously identified as a PKA-sensitive phosphorylation site important for G_s-stimulation of adenylyl cyclase (*31*).

In the absence of vanadate, the extent of forskolin-stimulated adenylyl cyclase activity [as well as Gpp (NH)p and PGE_1-stimulated adenylyl cyclase activity] in each of the flag AC6 mutants was indistinguishable from that in wild-type flag AC6 (Figure 5C and Table 2), demonstrating that the serine mutations do not affect the normal function of AC6. However, vanadate stimulation failed to induce sensitization of adenylyl cyclase activity for mutants A and B, but not for the S588A mutant (Figure 5C). Consistent with a role of p74^{raf-1}-mediated serine phosphorylation of the C_{1b} and IC4 domains of AC6, mutants A and B were resistant to vanadate-stimulated phosphorylation, whereas S588A mutant was effectively phosphorylated (Figure 5D). The differences in functional activity and phosphorylation state were not explainable by alterations in the extent of protein expression or cell surface expression, since all of the flag AC6 mutants were expressed at similar levels in plasma membrane-enriched fractions of HEK 293 cells (Figure 5E).

The comparable attenuation of vanadate-mediated responses seen in mutants A and B suggests that both of these closely associated domains (C_{1b} and IC4) are required to sustain tyrosine kinase-dependent enhancement of AC6 activity. Furthermore, the integrity of both domains is essential for phosphorylation suggesting the need for a domain-specific conformation required for p74^{raf-1} association in response to tyrosine kinase activation.

Adenylyl cyclase phosphorylation leading to regulation of activity has been described for many of the adenylyl cyclase isoforms. These effects have been mediated exclusively by serine/threonine kinases—primarily those activated by GPCRs (Table 3). Tyrosine kinase-mediated regulation of adenylyl cyclase does appear to utilize an analagous mechanism (i.e., acting via serine phosphorylation). However, the pathway by which this serine kinase is activated (i.e., raf-1-dependent) or the molecular domains of AC6 involved were previously unknown.

In summary, these data are consistent with an important role for p74^{raf-1} in the phosphorylation-dependent sensitization of adenylyl cyclase which occurs in response to the activation of tyrosine kinase signal transduction pathways (Figure 6). These data further underscore the complexity of the web of interactions between adenylyl cyclase- and p74^{raf-1}-dependent pathways. The effect of adenylyl cyclase activation (via PKA) to inhibit of p74^{raf-1}-mediated responses has been previously appreciated (*39*, *40*). The role of p74^{raf-1} activation on adenylyl cyclase response has not. However, in view of the very significant impact (quantitatively) of tyrosine kinase activation on cAMP synthesis, this pathway may be seen as a key regulator of adenylyl cyclase function. Adenylyl cyclase has been dubbed a "coincidence detector" integrating concurrent signals from Gs, Gi, and more recently NO-linked pathways (*41*). The current studies add an additional level of complexity to the regulation of adenylyl cyclase function.

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