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Isoform specific regulation of adenylyl cyclase function by disruption of membrane trafficking

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Running title: Regulation of adenylyl cyclase trafficking

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ABSTRACT

Oligomerization plays an important role in ER processing and membrane insertion (and ultimately in regulation of function) of a number of transmembrane spanning proteins. Further, it is known that adenylyl cyclases (AC), critical regulators of cellular functions, associate into higher order (dimeric) forms. However, the importance of these higher order aggregates in regulating adenylyl cyclase activity or trafficking to the cell membrane is unclear. Therefore, we examined the potential role of oligomerization in the membrane trafficking of adenylyl cyclase. For this purpose the ability of full-length adenylyl cyclase and various truncation mutants to self-assemble and to be targeted to the cell membrane was assessed. A truncation mutant comprised of the initial six transmembrane spanning domains and half of the C1 catalytic domain, co-immunoprecipitated with full-length AC VI. Utilizing both biotinylation assays and assessment of enzyme distribution using sucrose density gradients, we demonstrate that expression of this mutant in HEK293 cells impaired the ability of AC VI to traffic to the plasma membrane. Further, mutant expression resulted in a significant reduction in adenylyl cyclase activity. The decrease in AC VI membrane expression was not due to alterations in enzyme transcription. The effect of the mutant was specific for the AC V and VI isoforms and expression of the TM1 domain but not the C1a domain was required for the mutant to affect adenylyl cyclase activity. In aggregate, these data suggest that alterations in the ability of adenylyl cyclases to form higher order forms regulate both enzyme trafficking and enzyme activity.

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INTRODUCTION

Adenylyl cyclases comprise a large family of enzymes that catalyze the synthesis of cyclic AMP from ATP. Adenylyl cyclases constitute the "effector" component of transmembrane signaling pathways that are both positively and negatively regulated by the activity of heterotrimeric G-protein and G-protein coupled receptors (GPCRs). The activity of the enzyme adenylyl cyclase is itself regulated by both extracellular stimuli (via GPCR receptor activation) and intracellular stimuli (Cooper et al., 1995; Hanoune and Defer, 2001; Patel et al., 2001). Studies of the regulation of adenylyl cyclases initially focused "upstream" of the enzyme (i.e., on characterization of regulation of enzyme function via G-protein and GPCR function/expression) or on the regulation of substrate (Mg^{2+}/ATP) availability. More recently, the importance of regulation of enzyme activity by covalent modifications (i.e., enzyme phosphorylation) has increasingly been appreciated (Cooper, 2003; Hurley, 1998; Hurley, 1999; Patel et al., 2001; Tang and Hurley, 1998). However, regulation of adenylyl cyclase function via regulation of translation and trafficking to the plasma membrane has been largely unexplored. Further, the determinants of adenylyl cyclase trafficking to the cell membrane are unknown.

Structurally, the adenylyl cyclase enzyme consists of two hydrophobic domains (M1 and M2, containing six transmembrane spans each) and two large cytoplasmic domains (C1 and C2). The C1 and C2 regions are further subdivided in to "a" and "b" regions. The C1a and C2a regions interact to form the catalytic domain of adenylyl cyclase (Ludwig and Seuwen, 2002; Tang and Hurley, 1998). The transmembrane spanning domains have been suggested to be important in the regulation of protein assembly and membrane trafficking of the enzyme (Gu et al., 2001) .

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It is known that for a number of other transmembrane spanning proteins, including GPCRs, that oligomerization plays an important role in endoplasmic reticulum processing and ultimately plasma membrane insertion (Balasubramanian et al., 2004; Kaykas et al., 2004; Salahpour et al., 2004) . However, whether regulation of the formation of higher order aggregates of adenylyl cyclase might regulate plasma membrane insertion (and ultimately enzyme activity) is unclear.

Previous studies have identified that some adenylyl cyclase isoforms can be identified as higher order aggregates (Haga et al., 1977; Neer et al., 1984; Nielsen et al., 1981; Schlegel et al., 1979; Smigel, 1986; Yeager et al., 1985). Further, disruption of adenylyl cyclase dimerization has been reported to lead to impairment of enzyme activity. Specifically, expression of an inactive truncated form of adenylyl cyclase type VI has been demonstrated to reduce adenylyl cyclase type VIII function (Gu et al., 2002). However, the mechanism underlying this effect and the specific domains that determine this interaction are unclear. Further the isoform-specificity of this effect is unknown.

Therefore we tested the hypothesis that membrane expression of adenylyl cyclases could be regulated by disruption of membrane trafficking of the enzyme-- utilizing truncation mutants of the enzyme. Data to be presented demonstrate that expression of truncation mutations of AC VI including the transmembrane M1 (TM1) region can associate with wild type adenylyl cyclase and inhibit adenylyl cyclase function on an isoform-specific basis- primarily by interfering with the incorporation of the enzyme in the plasma membrane. These findings support a critical role of association of oligomers of adenylyl cyclase in cytoplasmic compartments in the trafficking of the enzyme and ultimately in the isoform-specific regulation of enzyme function.

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MATERIALS AND METHODS

Cell culture

HEK293 cells (human embryonic kidney cells, CRL-1573, American Type Culture Collection, Rockville, MD) were grown in Minimum Essential Medium (MEM, Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS, Hyclone Laboratories Inc) and 50 μg/mL gentamicin (Gibco). Cells were maintained at 37°C, in a humidified 5% CO_2 incubator.

Construction of truncation mutants

We initially generated four adenylyl cyclase type VI truncation mutations, NT (comprising the cytoplasmic N-terminal domain), C1aT (comprising the C1a domain of the enzyme), TM1T (comprising the N terminal domain and first 6 transmembrane spanning domains) and 547T (comprising the first 547 amino acids of the enzyme including the initial transmembrane spanning domain and the C1a domain) (Figure 1A), to examine the effects of co-expression on wild-type (full-length) adenylyl cyclase VI activity. pIND-AC VI truncation mutants (C1aT, NT, TM1T, 547T) were constructed by PCR using pIND-flag AC VI as template and primers as indicated in Table 1 and Figure 1A. PCR fragments were digested with HindIII/XbaI, and then cloned into pIND vector, which had been digested with identical enzymes. Positive clones were sequenced to verify the right open reading frame and right mutation**.** All truncation mutants were flag-tagged and were all detected in whole cell lysates from transfected cells (Figure 1B).

In addition, we subsequently generated a TM2T truncation mutant of AC VI and a TM1 truncation mutant of AC I. The TM2T truncation mutant of AC VI comprised the C1b, the second transmembrane spanning domain and C2 regions of AC VI. Flag-tagged TM2T was generated by PCR using flag-AC VI as template (Table 1). We also generated an AC I truncation

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mutant, 462T analogous to the 547T mutant of AC VI. The flag-tagged AC I 462T was generated by PCR using pcDNA-flag-AC I as template (Table 1). PCR fragment was digested with BamI/XbaI and cloned into pIND vector digested with identical enzymes.

Construction of myc-tagged adenylyl cyclase VI

Myc-AC VI was generated by PCR using flag-AC VI as a template. Using a forward primer containing myc tag (5'-CCC **AAG CTT**(HindIII) GCC GCC ATG GAG CAG AAA CTC ATC TCT GAA GAG GAT CTG CCC CTG-3'), and reverse primer GC TCT AGA CTA ACT GCT GGG GCC CCC ATT GAG-3'. PCR fragment was digested with HindII/XbaI, and cloned into pIND vector digested with identical enzymes.

Transfections

Transfections of HEK293 cells were carried out using a modification of the calcium phosphate method as previously described (Cullen, 1987). Cells were transiently co-transfected 3 μg of (pIND) flag-AC VI and 3 μg of either (pIND) GFP, (pIND) myc-AC VI or adenylyl cyclase mutants with 6 μg of pVgRXR. For isoform experiments co-transfection of 3μg of (pIND) flag-AC isoforms (AC I, AC II, AC V or AC VI), with 3 μg of either (pIND) GFP or truncation mutants plus 6 μg of pVgRXR was used. Following 24hr of transfection, expression of inserts was induced by addition of ponasterone A to final concentration to 5 μmol/L at 37°C for an additional 24 hours.

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Assessment of adenylyl cyclase activity

Adenylyl cyclase activity in response to forskolin (100 μM) or forskolin plus vanadate (300 μM) was assessed by the conversion of [α^{32}P] ATP to [^{32}P]-cAMP as previously reported (Tan et al., 2001). Briefly, permeabilized cells were resuspended in a solution of Hanks Balanced Salt Solution with 33 mmol/L HEPES, 0.5 mmol/L EDTA and 1 mmol/L magnesium sulfate (pH 7.4 at 4^{o}C) were added in an aliquot of 40 μL to give a final incubation volume of 100 μL with 1 μCi [α^{32}P] ATP, 0.3 mmol/L ATP, 2 mmol/L $MgSO_4$, 0.1 mmol/L cAMP (used in lieu of a phosphodiesterase inhibitor), 5 mmol/L phosphoenol pyruvate, 40 μg/ml pyruvate kinase and 20 μg/ml myokinase. Incubations were carried out at 37°C for 10 minutes and terminated by addition of 1 ml of a solution containing 100 μg ATP, 50 μg cAMP and 15,000 cpm [^{3}H] cAMP. Cells were pelleted by centrifugation at 300x g for 5 minutes. cAMP was isolated from the supernatant by sequential Dowex and alumina chromatography and was corrected for recovery with [^{3}H] cAMP as the internal standard. Adenylyl cyclase activity was linear with time and cell number over the ranges used. Adenylyl cyclase activity was expressed as percentage of control-transfected cells. In experiments examining the effects of various truncations mutants on AC isoforms (ACx), activity was expressed as a percentage of ACx-alone transfected cells (i.e. AC VI plus 547T expressed as a percentage of AC VI alone transfected cells, (AC VI + 547T)/ AC VI). In experiments where truncation mutants were transfected alone, the activity was expressed as a percentage of activity in GFP-transfected cells.

Immunoprecipitation and Western blotting

Transfected cells were lysed in buffer A (20 mM Tris, pH 8.0, 1% NP-40, 0.1% SDS, 140 mM NaCl, 1 mM phenylmetanesulfonyl fluoride). For co-immunoprecipitation experiments, 500 μg

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of cell lysates were incubated with 5 μL of an anti-adenylyl cyclase "AC comm" polyclonal antibody that demonstrates cross-reactivity with many adenylyl cyclase isoforms (the epitope was generated against a 14 amino acid peptide of the c-terminal region of adenylyl cyclase, found to be similar in several cloned adenylyl cyclase isoforms (Pieroni et al., 1995)) for 1 hour at 4°C followed by the addition of 25 μL of A/G agarose beads (Santa Cruz Biotechnology, CA) and further incubated overnight at 4°C on a rocker platform. The resulting immunoprecipitation complexes were resolved on SDS-PAGE and blotted as described below.

Sixty micrograms of whole cell extracts were resolved on SDS-PAGE, were blotted electropheretically onto Immun-Blot PVDF membrane (Bio-Rad, Hercules CA). Membranes were blocked with 5% skim milk and incubated with anti-flag (M2) antibody (1:1000, Sigma St. Louis MO) overnight at 4°C on a rocker platform. Blots were washed in tris-buffered saline for 1 hour followed by incubation of secondary anti-mouse antibody (1:1000, Sigma) for 1h at room temperature. Proteins were detected by chemiluminescence as described by the manufacturer's protocol (NEN, Boston, MA).

Cell Surface Biotinylation

Transfected cells were induced for 24h with 5 μmol/L ponasterone A. Cells were washed with cold-PBS three times to remove contaminating proteins, then biotinylated in the presence of 0.5 mg/ml sulphosuccinimidyl-2- (biotinamido) ethyl-1, 3-dithiopropionate (EZ-link sulfo-NHS-SS-biotin; Pierce Chemical Co.) for 30 min at 37°C. Cells were washed with PBS containing 0.1% BSA and then solubilized in lysis buffer. Biotinylated proteins were precipitated by streptavidin-agarose (Sigma) and separated on SDS-PAGE, transferred onto PVDF membrane and immunoblotted with anti-flag antibody as described above.

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RT-PCR Assessment of AC VI mRNA

Total RNA was extracted from HEK293 cells transfected with AC VI alone or with truncation mutant 547T by Trizol reagent (Invitrogen Canada Inc.). cDNAs were generated by reverse transcription of RNA using SuperScript kit (Invitrogen Canada Inc.) and used as template for PCR reactions with the following oligonucleotide primer pairs: 5'-CCTGGCCAAGGTCATCCATGACAACT-3' and 5'-TGTCATACCAGGAAATGAGCTTG-3' for the internal control GAPDH, and 5'-GCGCAACGCGTACCTCAAGGA-3' and 5'-TCTTCTCGAGATCCTCCCTCTGGAAGG-3' for AC VI. Standard PCR was performed with Taq DNA polymerase for 30 cycles. The resulting PCR products were resolved by agarose electrophoresis and visualized by ethidium bromide staining.

RNA Protection Assays

Total RNA was isolated 24h post-ponasterone A induction from HEK293 cells transfected with AC VI alone or with truncation mutant 547T. RNA protection assay was performed using an Ambion RPAII kit with hybridization at 42°C overnight according to the manufacturer's procedure; $2x10^4$ cpm of AC VI (1651-2010) or GADPH riboprobe was added to 10 μg of HEK293 RNA, and the RNA-RNA hybrids which resisted RNAse treatment were precipitated, dissolved in loading buffer and denatured at 95°C for 5min. Components then were resolved on a 4% polyacrylamide-8M urea denaturing gel in TBE buffer. After drying, gels were exposed to X-ray films at -70°C with intensifying screens.

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Sub cellular fractionation.

HEK293 cells expressing AC VI alone or co-expressing AC VI plus the truncation mutation 547T were detached in ice-cold PBS containing 2 mM EDTA, cells were pelleted and resuspended in homogenization buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, and 0.25 M sucrose, supplemented with protease inhibitor mixture of 1 mM phenylmetanesulfonyl fluoride, with 1 μg/ml each of antipain, leupeptin and pepstatin A. The cells were disrupted using 10 strokes in Dounce homogenizer followed by four passages through a 25-gauge needle. Nuclei and unbroken cells were pelleted by centrifugation at 3000x g for 10min. The pellet was resuspended in 1 ml of homogenization buffer and centrifuged at 3000x g for 10min. Post nuclear supernatants from two centrifugation steps were combined and centrifuged at 80,000x g for 1h. The vesicle pellet was resuspended in 1ml of homogenization buffer and loaded on top of 1-20% iodixanol continuous linear gradients and centrifuged in Beckman SW 41 rotor at 200,000x g for 3h at 4°C. Sequential 0.85 ml fractions were collected from the bottom of the gradient. The sub cellular fractions were lysed by the addition of sample buffer and resolved by 8% SDS-PAGE, and immunoblotted with anti-flag or a plasma membrane marker antibody (viz., anti-Na^+/K^+-ATPase, Santa Cruz Biotechnology, CA).

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RESULTS

To test the hypothesis that membrane expression of adenylyl cyclases could be regulated by disruption of membrane trafficking of the enzyme we sought to initially determine whether expression of the truncation mutants of the enzyme regulated a) enzyme activity and b) membrane insertion of functional enzymes. To examine the functional effects of adenylyl cyclase truncation mutants on adenylyl cyclase activity, we transfected HEK293 cells with either ecdysone-inducible plasmids for GFP, AC VI alone or AC VI co-transfected with one of the four-adenylyl cyclase VI truncation mutants (Figure 1A). All truncation mutants were detectable by immunoblots in whole cell lysates from transfected cells (Figure 1B). Expression of wild type AC VI alone significantly increased forskolin-stimulated adenylyl cyclase activities as compared to GFP-transfected cells (163±9% of activity in GFP-transfected cells, n=13, $p<0.05$). Co-expression of either 547T- or TM1T-truncation mutants with AC VI significantly inhibited forskolin-stimulated adenylyl cyclase activity as compared to AC VI-alone transfected cells (Figure 2). Also, expression of the 547T mutant alone significantly inhibited endogenous adenylyl cyclase activity by 16±3% (n=14, $p<0.05$) as compared to GFP-transfected cells. In contrast, co-expression of the C1aT or NT -truncation mutants with wild type AC VI did not significantly alter forskolin-stimulated adenylyl cyclase activation (Figure 2).

To determine whether the effect of these mutants to alter adenylyl cyclase activity might be via disrupting membrane insertion/trafficking we performed cell surface biotinylation studies. When either expressed individually or together with full-length adenylyl cyclase VI, biotinylated forms of the TM1T and 547T-truncation mutants as well as wild type adenylyl cyclase could be identified (Figure 3A). However, co-expression of either the 547T or TM1T mutants with wild type AC VI resulted in a significant reduction in the biotinylated monomeric form of adenylyl

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cyclase VI (Figure 3A and 3B). In contrast, biotinylated forms of the C1aT or NT were not detectable (data not shown) and did not alter the detection of biotinylated monomeric form of adenylyl cyclase VI (Figure 3A and 3B). Thus the ability of the TM1T and 547T mutants to inhibit adenylyl cyclase function (but not the C1aT or NT mutants) paralleled their ability to inhibit plasma membrane expression of full-length adenylyl cyclase VI. Notably, total cellular adenylyl cyclase VI expression in cells (as detected by expression of a 126 kDa protein which migrated identically with recombinant AC VI) was not altered with co-expression of the TM1T mutant (nor with the co-expression of either C1aT and NT mutants, Figure 4A and 4B). However, total cellular AC VI expression was significantly reduced with co-expression of the 547T truncation mutant (32±5% of AC VI alone transfected cells, n=6, $p<0.05$, Figure 4A and 4B).

To confirm that 547T expression decreased the *proportion* of plasma membrane:total cellular AC VI (given that both total cellular and cell surface AC VI expression was decreased), we quantitated the proportion of membrane-associated adenylyl cyclase VI with and without 547T co-expression using sucrose density centrifugation techniques (as determined by that proportion of the expressed adenylyl cyclase that co-fractionated with the plasma membrane marker, Na^+/K^+-ATPase). These studies demonstrated that co-expression of the 547T mutant significantly reduced the plasma membrane-associated expression of AC VI (73%±3% of AC VI alone, n=3, $p<0.05$).

To determine whether the apparent reduction in protein expression of wild-type AC VI observed with co-expression of 547T was due to decreased transcription of wild-type adenylyl cyclase, we performed both RT-PCR and RNase protection assays. RT-PCR revealed comparable detectable fragments of AC VI in RNA isolated from either cells transfected with

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both AC VI and the 547T truncation mutant or cells transfected with AC VI alone (AC VI+547T: 107±2% of AC VI alone, n=3, p=NS, Figure 5A). Similarly RNase protection assays revealed fragments of adenylyl cyclase VI that were not significantly different when co-expressed with the 547T mutant as compared to AC VI alone expressing cells (AC VI +547T: 128±18% of AC VI alone, n=3, p=NS, Figure 5B).

In aggregate, the reduction in total AC VI protein expression without evidence of alterations in AC VI transcription suggests an enhancement of AC VI degradation with 547T co-expression (beyond its effect to regulate enzyme function by impairing membrane insertion of full length adenylyl cyclase).

Previous studies have suggested that the ability of adenylyl cyclase isoforms to form higher order aggregates maybe important in regulation of enzyme function (Gu et al., 2002; Gu et al., 2001). Based on these observations, we next sought to more directly determine whether these truncation mutants might interfere with the homo-association of adenylyl cyclase molecules. Therefore, we first attempted to confirm that full-length AC VI molecules could directly associate into higher order aggregates. To do this, cells were transfected with either myc-tagged, flag-tagged or *both* myc- and flag-tagged full-length adenylyl cyclase VI. In co-immunoprecipitation studies, an anti-myc-immunoreactive 126 kDa band was observed following immunoprecipitation with anti-flag antibody in cells transfected with both myc- and flag-tagged full-length adenylyl cyclase VI (Figure 6A), but not in cells transfected with either flag- or myc-tagged AC VI alone (Figure 6A). Similarly, immunoprecipitation with anti-myc antibody followed by immunoblotting with anti-flag antibody resulted in the identification of a single flag-immunoreactive band of 126 kDa (Figure 6B). These findings are consistent with those in prior

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reports (Gu et al., 2002; Gu et al., 2001) suggesting that adenylyl cyclase VI molecules can form stable higher order complexes.

Based on our demonstration that full-length adenylyl cyclase VI molecules can form higher order aggregates, we next determined whether a direct interaction between these truncation mutants and full-length adenylyl cyclase VI might occur in co-transfected cells. We immunoprecipitated wild type AC VI from whole cell lysates using an anti-adenylyl cyclase antibody (AC comm, whose antibody epitope was based on the highly homologous carboxy terminal domain of adenylyl cyclases i.e., a domain not common to any of the four-truncation mutants). When co-expressed with full-length AC VI (Figure 7) both the TM1T and 547T-truncation mutants could be immunoprecipitated by this carboxy terminal-specific adenylyl cyclase antibody -- consistent with a direct interaction between wild type AC VI and these truncation mutations. In contrast, the NT and C1aT truncation mutants did not co-immunoprecipitate with full-length AC VI (Figure 7).

In aggregate these studies indicated that a) adenylyl cyclase VI could form higher order aggregates, and b) the ability to form heteromeric aggregates between full-length adenylyl cyclase and deletion mutations containing the TM1 domain of the enzyme paralleled their ability to impair plasma membrane insertion of adenylyl cyclase VI and enzyme function.

To determine whether the effect of the 547T mutant, based on the sequence of adenylyl cyclase VI, was specific for AC VI or was generalized to other isoforms, we examined the effect of co-expressing the 547T-mutant with other adenylyl cyclase isoforms representative of other families of the enzyme i.e., AC I, and AC II, as well as AC V (the other member of the ACVI family). Expression of AC I, AC II and AC V alone significantly increased forskolin-stimulated adenylyl cyclase activity as compared to GFP-transfected cells (AC I: 267±37% of GFP; AC II:

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267±35% of GFP, and AC V: 294±22% of GFP, n=9-13, $p<0.05$ for all isoforms) and comparably to that seen with ACVI (163±9% of GFP). The 547T mutant co-immunoprecipitated with all of the isoforms of adenylyl cyclase examined (Figure 8A). Co-expression of the 547T-mutant significantly reduced the extent of adenylyl cyclase activity seen with expression of AC VI and (to a lesser extent) AC V, but did not alter the extent of adenylyl cyclase activity observed with expression of either AC I or AC II (Figure 8B). Similarly, biotinylation experiments revealed that AC I cell-surface expression was not significantly affected by the co-expression of 547T truncation mutant (98±30% of biotinylation observed in AC I alone expressing cells, n=3). Thus, formation of these heteromeric aggregates might occur with all adenylyl cyclase isoforms but is not sufficient to disrupt membrane protein expression and consequently function of adenylyl cyclases.

Previous studies have demonstrated strong homomeric interactions between the second transmembrane clusters (TM2) of adenylyl cyclase (Gu et al., 2001), suggestive of higher order structures of adenylyl cyclase. Therefore, to determine the effect of TM2 on AC expression, we constructed a truncation mutation of adenylyl cyclase VI incorporating the M2 and C2 domains (the COOH-terminal half) of the enzyme. Co-expression of TM2T with either AC VI or with the other adenylyl cyclase isoforms did not significantly reduce adenylyl cyclase activity for any of the isoforms examined (AC I: 81±6% of control; AC II: 86±4% of control; AC V: 90±2% of control and AC VI: 82±13% of control, n=4, $p>0.10$ for all isoforms).

To examine whether the inhibition of adenylyl cyclase VI by a truncation mutant comprising the N-terminal half of adenylyl cyclase VI (the 547T mutant) was AC VI-specific (or would be generalized for truncation mutations constructed from other isoforms of adenylyl cyclase), we generated a similar N-terminal truncation mutant for adenylyl cyclase isoform I (AC

I 462T). In contrast to the effect of the AC VI N-terminal mutant (547T), co-expression of the adenylyl cyclase I N-terminal truncation mutant (mutant 462T) did not significantly alter adenylyl cyclase activity for any of the isoforms examined (AC I: 96±5% of control; AC II: 93±6% of control; AC V: 100±3 of control and AC VI: 85±5% of control, n=4, $p>0.10$ for all isoforms).

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DISCUSSION

Previous studies have suggested that the ability of adenylyl cyclase isoforms to form higher order aggregates were important in regulation of enzyme function (Gu et al., 2002; Gu et al., 2001). The present studies demonstrate that disruption of the ability of the enzyme to form effective higher-level aggregates is associated with impairment of the membrane expression of the enzyme and ultimately activity. We demonstrate that expression of a truncation mutation of AC VI impairs adenylyl cyclase function in both native cells and wild type AC VI expression models. This adenylyl cyclase VI-truncation mutation co-immunoprecipitated with wild type (full-length) AC VI and decreased the plasma membrane expression of the enzyme (as detected in biotinylation experiments). This effect was specific for truncation mutations containing the M1 domain of the enzyme (i.e. 547T and TM1T) and was not replicated by smaller truncated forms of the enzyme that did not contain the M1 region or by expression of a truncation mutant comprising the carboxy terminal half of the enzyme. The effect of this mutant was adenylyl cyclase isoform-selective-- i.e., the mutant did not affect the function or expression of representative isoforms of other adenylyl cyclase subfamilies. Additionally expression of an analogous truncation mutation of AC I did not replicate the effect of the truncation mutation of AC VI.

Inhibition of adenylyl cyclase activity has been previously been related to alterations in concentrations of ionic determinants of activity (i.e., either decreasing magnesium concentrations or increasing calcium concentrations), activation of inhibitory G-proteins or by covalent modification of the receptor (e.g., by PKA-mediated serine phosphorylation of the receptor) (Cooper, 2003; Hurley, 1998; Hurley, 1999; Patel et al., 2001; Tang and Hurley, 1998). However, the role of disrupting the functional assembly of the enzyme on its activity and the mechanism of

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this effect has been largely unexplored. It is notable that the importance of the cytoplasmic domains in regulating adenylyl cyclase activity has been extensively studied. Utilizing small synthetic peptides based on the sequence of the C1a region of adenylyl cyclase V sequence data, Kawabe and co-workers demonstrated inhibitory effects on adenylyl cyclase II and V (Kawabe et al., 1994). However, whether these effects were due to direct inhibition of enzyme activity or disruption of enzyme trafficking was not addressed.

The importance of the TM1 domains in impairing enzyme function by disrupting enzyme trafficking has been largely unexplored. Largely based on the study of adenylyl cyclase VIII, interactions between the **transmembrane** spanning domains have been suggested to be a critical determinant in the regulation of the enzyme. Gu and co-workers demonstrated that with adenylyl cyclase VIII expression, enzyme activity could be suppressed by co-expression of either an inactive adenylyl cyclase type VIII deletion mutant (deletion of amino acids 582-594) or an inactive adenylyl cyclase VI deletion mutant (deletion of amino acids 553-666). It is notable that these suppressive effects of the adenylyl cyclase type VIII deletion mutants were also apparent with expression of adenylyl cyclase isoforms V and VI. (Gu et al., 2002). In contrast, the current studies indicate that the TM1 domain is the critical determinant for efficient AC VI trafficking and this effect is specific in the contexts of a) the adenylyl cyclase domain, b) the adenylyl cyclase isoform from which the truncation protein was constructed and c) the adenylyl cyclase isoform(s) that are affected by expression of this mutant protein. The reason for the apparent variance of the present findings with those of Gu et al is unclear. However, it is notable that in their studies the apparent expression of adenylyl cyclase (as judged by the proportional increase in activity over sham-transfected cells) was much greater than in the current studies (~500% vs.

~170%). Thus whether the lack of isoform specificity of their constructs reflected the difference in expression levels remains to be determined.

To date, nine membrane-bound isoforms of adenylyl cyclase have been cloned and characterized in mammalian tissues and/or cells (Defer et al., 2000). The overall structure is similar between all membrane-bound isoforms. However, despite the similarities in structure and function of the adenylyl cyclase isoforms, it has been increasingly appreciated that their regulation and tissue-specific expression varies considerably (Cooper, 2003) (Defer et al., 2000; Hanoune and Defer, 2001). Previously differences between isoforms have been demonstrated in the context of regulation by G-proteins, ionic concentrations and via differences in the patterns of covalent modification of the enzyme (primarily via phosphorylation) (Cooper, 2003; Hurley, 1998; Hurley, 1999; Patel et al., 2001; Tang and Hurley, 1998). The current studies identify another potentially important isoform-specific mechanism of regulation. The effect of the 547T-truncation mutant of AC VI did have effects on AC V function. However, this effect was not seen for representative isoforms from the two other major subfamilies. There is very strong sequence homology between AC V and AC VI (> 80% (Hanoune et al., 1997))- thus a common effect would not have been unexpected. However, somewhat surprisingly, the other AC isoforms studied co-immunoprecipitated with the mutant, although the mutant did not alter their functions. Thus, heteromeric association (as detected by this approach) might occur, but is clearly not sufficient to account for the disruption of function. Whether this reflects quantitative differences in affinity for heteromeric association or the importance of additional interaction sites not common to these other isoforms remains to be determined.

Prior studies have focused on disrupting enzyme activity by impairing activity of the membrane-bound protein. The current studies suggest a novel approach for regulating function

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by disrupting enzyme association in cytoplasmic compartment-- probably by disrupting the formation of higher order aggregates of adenylyl cyclase. *Intramolecular* dimerization of adenylyl cyclase between the two transmembrane spanning domains has been suggested to be important for the association of C1 and C2 domains necessary for catalytic activity and G-protein regulation of the enzyme. Older literature had suggested the possibility of adenylyl cyclase dimerization. Specifically, earlier reports had indicated that detergent-solubilized adenylyl cyclase preparations displayed molecular masses over 200 kD-- suggestive of higher order complexes or dimerization of adenylyl cyclase (Haga et al., 1977; Neer et al., 1984; Nielsen et al., 1981; Schlegel et al., 1979; Smigel, 1986; Yeager et al., 1985). More recently, the ability of adenylyl cyclase isoforms to dimerize has been demonstrated by co-immunoprecipitation and FRET analysis (Gu et al., 2002; Gu et al., 2001). However, the importance of this association for either enzyme activity or for trafficking was unclear. Based on the current studies we would suggest that formation of homologous dimers is critical in regulation of enzyme trafficking. Further this might be a mechanism for ensuring the fidelity of expression of these membrane proteins, if the hetero-oligomerization of "defectively" translated adenylyl cyclases failed to be processed appropriately for trafficking to and insertion in the cell membrane.

It is important to note that our data do not rule out a role for more complex aggregates in either the membrane processing of adenylyl cyclases or ultimately in regulating enzyme function. Adenylyl cyclase isoforms have been shown to be a component of lipid rafts (Ostrom et al., 2004). Thus whether our findings are best accounted for by disruption of a more "simple" higher order protein aggregate or of a more complex lipid raft structure has not been elucidated.

Notably, in GPCR signal transduction systems, the concept that protein dimerization in the endoplasmic reticulum might be an important determinant in regulating their trafficking and

ultimately their expression and function in the plasma membrane is not novel. A similar mechanism has been implicated as a means of regulating fidelity of expression of G-protein coupled receptors such as the $beta_2$-adrenoceptor (Salahpour et al., 2004), the Frizzled receptor (Kaykas et al., 2004) and the GABAb receptor (Balasubramanian et al., 2004). Further, an expanding list of plasma membrane proteins including the insulin receptor (Bass et al., 1998),TGFβ receptors (Gilboa et al., 1998), AMPA calcium channels (Greger et al., 2002) and shaker potassium channels (Papazian, 1999) have been shown to oligomerize in endoplasmic reticulum compartments. For these proteins oligomerization has been suggested to be an important step in cell surface trafficking- perhaps relating to enhancing their association with molecular chaperones critical in protein processing (Yuan et al., 2003). Our findings suggest that adenylyl cyclase membrane insertion may be regulated similarly.

Whether regulation of adenylyl cyclase trafficking is an important determinant of adenylyl cyclase function either physiologically (or pathologically) is unknown. In addition, what role truncated adenylyl cyclase molecules may play in regulating adenylyl cyclase trafficking and hence adenylyl cyclase function is unclear. A truncated form of adenylyl cyclase VI, consisting of the first half of the molecule, was previously cloned from a canine cardiac cDNA library (Katsushika et al., 1993). However, what role this truncated adenylyl cyclase VI mutant plays *in vivo* is unknown. In addition, a novel isoform of adenylyl cyclase (DAC78C) was recently identified in *Drosophila melanogaster* (Cann and Levin, 2000). This adenylyl cyclase was similar to mammalian transmembrane adenylyl cyclase isoforms. Interestingly, this adenylyl cyclase was expressed as two structurally distinct proteins (one full-length and one truncated) in a temporal and tissue/cellular specific pattern (Cann and Levin, 2000). What role this truncated adenylyl cyclase plays in the embryogenesis of *D. melanogaster* is unknown.

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Thus, whether similar truncated adenylyl cyclases occur and/or play a role in mammalian cellular regulation of adenylyl cyclase is unknown, but could represent an unappreciated mechanism for regulation of adenylyl cyclase action.

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Footnotes

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FIGURE LEGENDS

Figure 1. Flag-tagged adenylyl cyclase VI and truncations mutants. Panel (A) schematic depiction of flag-tagged full-length adenylyl cyclase VI and four-truncation mutants. Panel (B). Detection of four truncation mutants on western blot. Whole cell lysates of transfected cells were immunoblotted with anti-flag antibody.

Figure 2. Effect of adenylyl cyclase VI truncation mutants on forskolin-stimulated adenylyl cyclase activity. Activity is represented as a percentage of forskolin-stimulated adenylyl cyclase activity from adenylyl cyclase VI alone-transfected cells. Both the 547T and TM1T truncation mutants significantly inhibited adenylyl cyclase activity. Data represent the mean±SEM from six independent experiments performed under identical conditions. *$p<0.05$ vs. AC VI alone transfected cells.

Figure 3. Significant reduction in membrane insertion/trafficking of adenylyl cyclase VI with co-expression of either 547T or TM1T truncation mutants. To assess the amount of adenylyl cyclase VI associated with the membrane, cell surface biotinylation experiments were performed. Panel (A), depicts the detection of anti-flag immunoreactive proteins following immunoprecipitation with streptavidin-agarose beads. Panel (B) densitometric analysis of biotinylated monomeric adenylyl cyclase VI in the absence and presence of 547T, TM1T, NT or Ca1T truncation mutants. Data represent the mean±SEM from 4-8 independent experiments performed under identical conditions. *$p<0.05$ vs. AC VI alone transfected cells.

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Figure 4. Assessment of whole cell adenylyl cyclase VI expression in the absence and presence of various truncation mutants. Panel (A) western blot of whole cell lysates immunoblotted with anti-flag. Panel (B) densitometric analysis of western blot expression of adenylyl cyclase VI (126 kDa) in the absence and presence of truncation mutants. The 547T truncation mutant significantly reduced the expression of a 126 kDa (monomeric adenylyl cyclase) species. Data are expressed as percent of AC VI expression (in AC VI alone transfected cells) and represent the mean±SEM for six independent experiments performed under identical conditions. *$p<0.05$ vs. AC VI alone transfected cells.

Figure 5. The effect of the 547T truncation mutant on transcription of adenylyl cyclase VI. Transcription of adenylyl cyclase VI was assessed via RT-PCR (Panel A) or RNase protection assays (Panel B). These data indicate that the reduction of adenylyl cyclase VI protein expression is not due to inhibition of transcription. The blots depicted are representative of the finding from three independent experiments performed under identical conditions.

Figure 6. Assessment of full-length adenylyl cyclase interactions. Cell were transfected with flag-AC VI alone, myc-AC VI alone or both flag-AC VI and myc-AC VI. Whole cell lysates were immunoprecipitated with either anti-flag antibody (Panel A) or anti-myc antibody (Panel B). Immunoprecipitates were then resolved and immunoblotted with either anti-myc antibody (Panel A) or anti-flag antibody (Panel B). The blots depicted are representative of the findings from three independent experiments performed under identical conditions.

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Figure 7. Assessment of adenylyl cyclase VI interactions with truncation mutants. Whole cell lysates were immunoprecipitated with anti-AC comm antibody, resolved and immunoblotted with the anti-flag antibody. TM1T and 547T truncation mutants could be co-immunoprecipitated with full-length adenylyl cyclase VI. In contrast, neither NT nor C1aT were co-immunoprecipitated with adenylyl cyclase VI. Note the lack of flag-immunoreactive bands when either TM1T or 547T truncation mutants when expressed alone. The blot depicted is representative of the findings from five independent experiments performed under identical conditions.

Figure 8. Assessment of co-immunoprecipitation (Panel A) and effect on forskolin-stimulated adenylyl cyclase activity (Panel B) of 547T truncation mutant on adenylyl cyclase isoforms. The 547T mutant co-immunoprecipitated with all the isoforms of adenylyl cyclase examined (Panel A), whole cell lysates were immunoprecipitated with anti-AC COMM antibody, resolved and immunoblotted with anti-flag antibody. Panel B, effect of 547T truncation mutant on forskolin-stimulated adenylyl cyclase activity in cells expressing AC I, AC II, AC V and AC VI. There was a significant effect of the 547T mutant on ACVI and ACV but not AC I, or AC II activity. Data represent the mean±SEM from five-ten independent experiments performed under identical conditions. *$p<0.05$ vs. ACx alone transfected cells.

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Table I. Primer sequence for construction of adenylyl cyclase truncation mutants.

AC VI NT (1-163aa)

Fp (ACF2) 5'-TC**AAGCTT(HindIII)**ATGGACTACAAGGACGACGAT-3'

rp 5'-GC**TCTAGA(XbaI)**CTAGCTGCTCTGGTTCATCTGGAAG-3'

AC VI TM1T (1-319aa)

fp(ACF2)5'-TC**AAGCTT(HindIII)**ATGGACTACAAGGACGACGAT-3'

rp 5'-GC**TCTAGA(XbaI)**CTAGTGCGTGCAGACACCGATGGCAT-3'

AC VI 547T (1-547aa)

fp(ACF2)5'-TC**AAGCTT(HindIII)**ATGGACTACAAGGACGACGAT-3'

rp 5'-GC**TCTAGA(XbaI)**CTAACGGCCTGGCTCCACCTCATAG-3'

AC VI C1aT (320-547aa)

fp 5'-CCC**AAGCTT(HindIII)**GCCACCATGGATTACAAGGATGACGACGATAAGTACCCCGCTGAAGTGTCTCAG-3'

rp 5'-GC**TCTAGA(XbaI)**CTAACGGCCTGGCTCCACCTCATAG-3'

AC VI TM2T (548-1181aa)

fp 5'-CCC**AAGCTT(HindIII)**GCCACCATGGATTACAAGGATGACGACGATAAG GGC GGTGAG CGCAACGCGTAC -3'

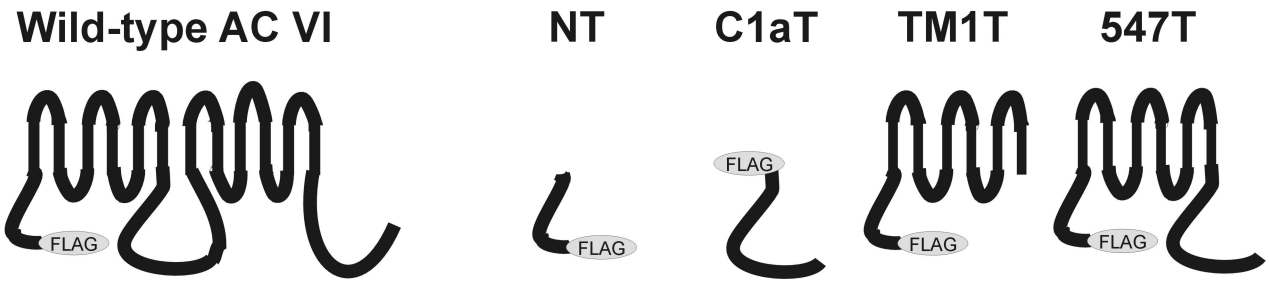
rp 5'-GC**TCTAGA(XbaI)**CTAACTGCTGGGGCCCCCATTGAG-3'

AC I 462T (1-462aa)

fp 5'-CG**GGATCC(BamHI)**GCCACCATGGATTACAAGGATGACGAC -3'

rp 5'GC**TCTAGA(XbaI)**CTAGTGTCCGTGTCCCGGCTCCACCT-3'

A


Wild-type AC VI
NT
C1aT
TM1T
547T
FLAG
FLAG
FLAG
FLAG
FLAG

B

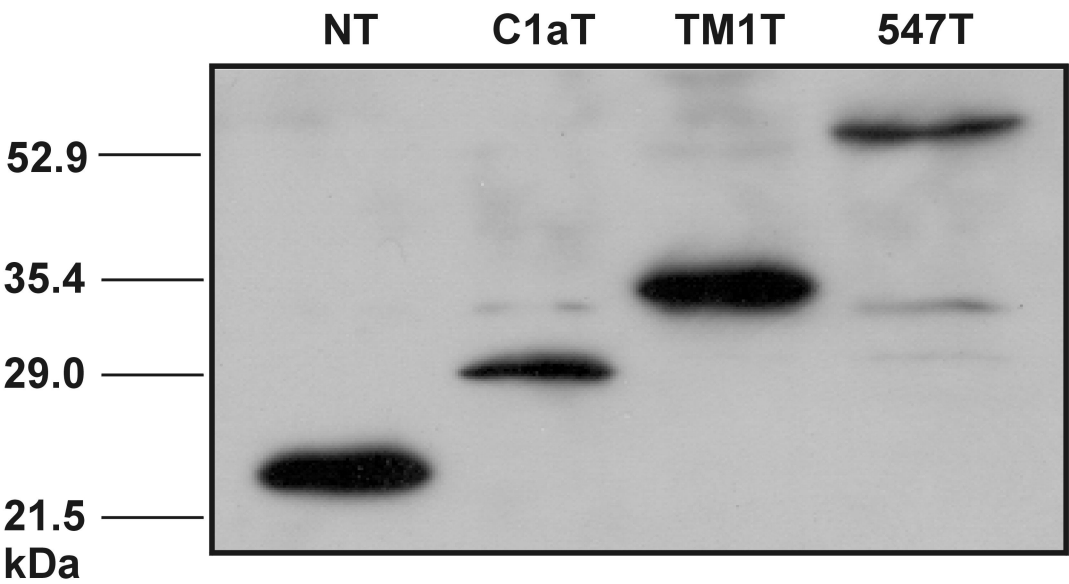

NT
C1aT
TM1T
547T
52.9
35.4
29.0
21.5
kDa

Figure 1

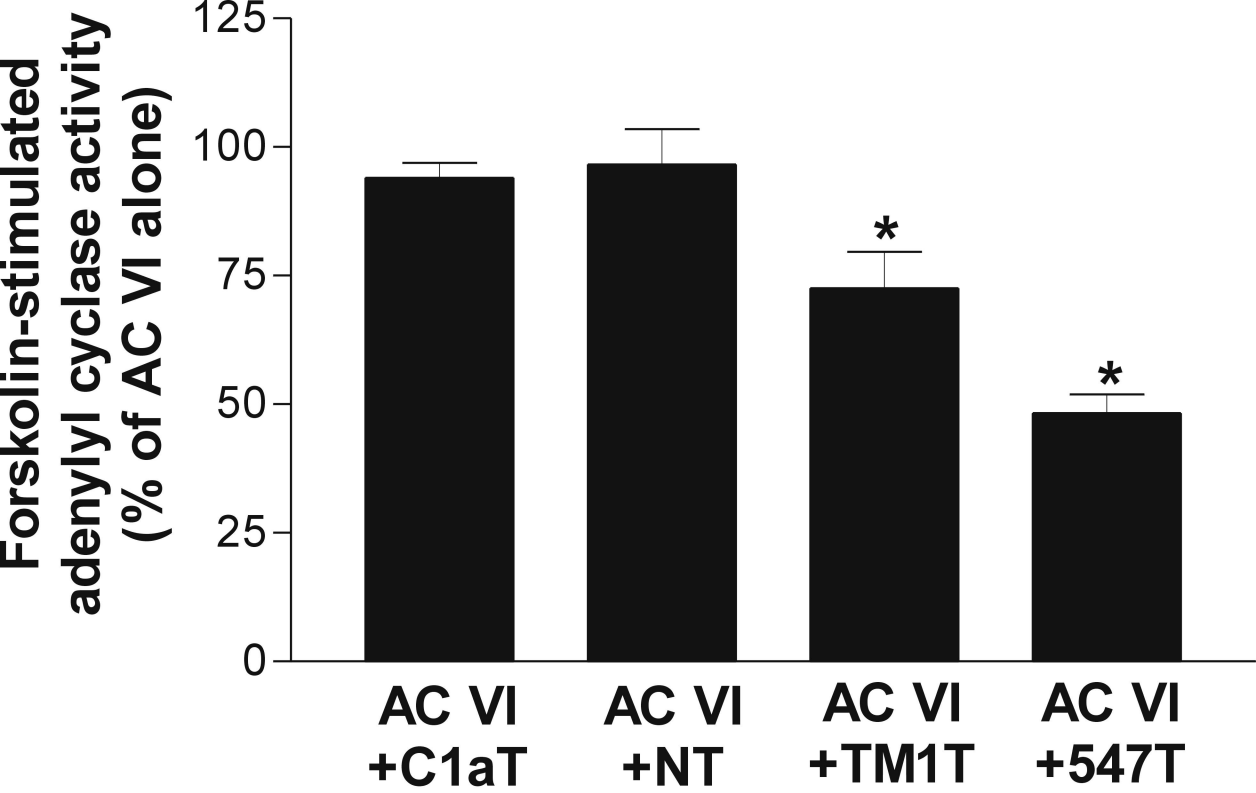

Forskolin-stimulated adenylyl cyclase activity (% of AC VI alone)
125
100
75
50
25
0
*
*
AC VI +C1aT
AC VI +NT
AC VI +TM1T
AC VI +547T

Figure 2

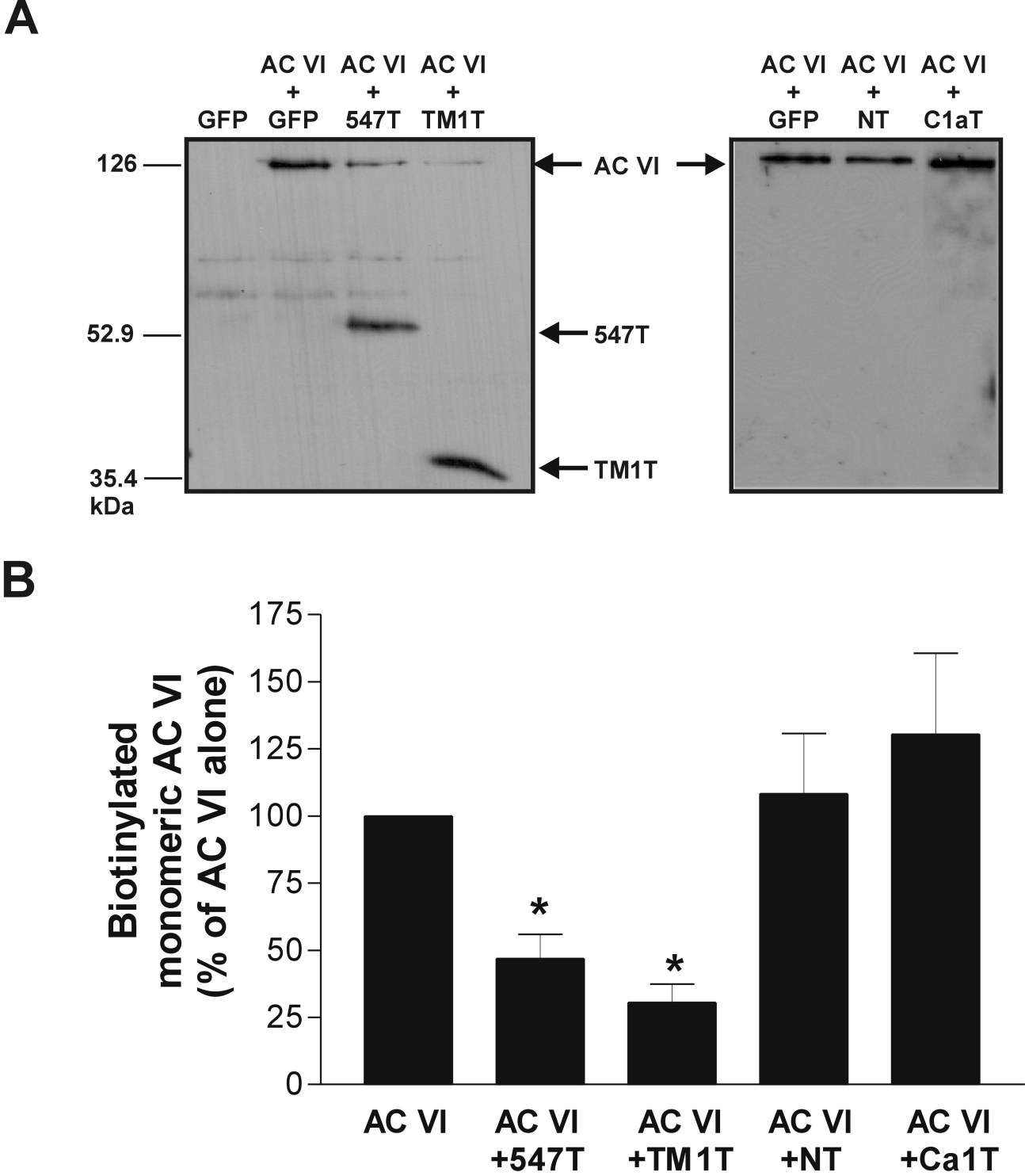

A
AC VI + GFP
AC VI + GFP
AC VI + 547T
AC VI + TM1T
AC VI + GFP
AC VI + NT
AC VI + C1aT
126
52.9
35.4 kDa
AC VI
547T
TM1T
B
Biotinylated monomeric AC VI (% of AC VI alone)
175
150
125
100
75
50
25
0
*
*
AC VI
AC VI +547T
AC VI +TM1T
AC VI +NT
AC VI +Ca1T

Figure 3

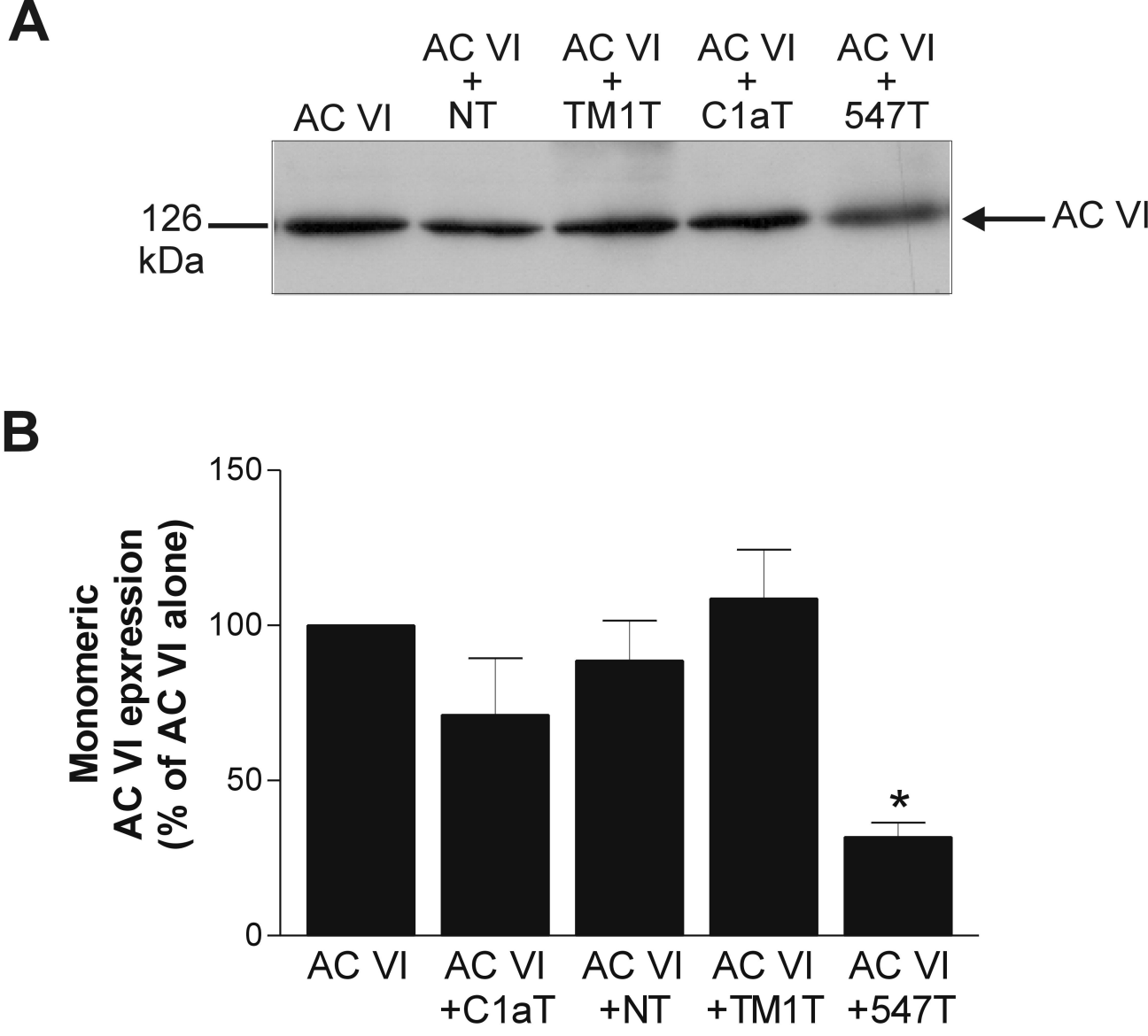
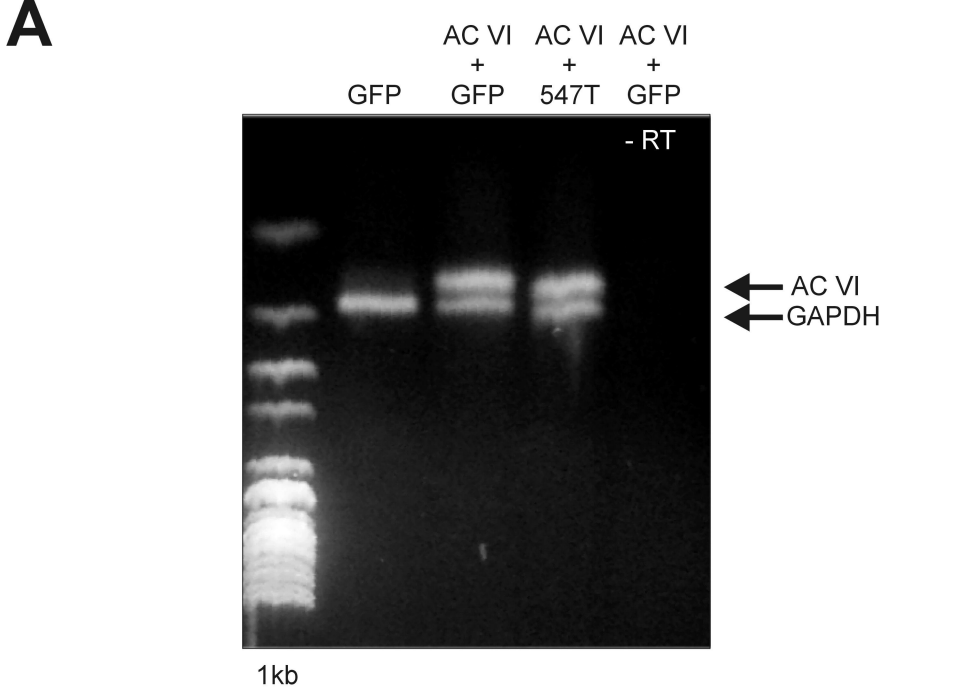

A
AC VI
AC VI + NT
AC VI + TM1T
AC VI + C1aT
AC VI + 547T
126 kDa
AC VI
B
Monomeric AC VI epxression (% of AC VI alone)
150
100
50
0
*
AC VI
AC VI +C1aT
AC VI +NT
AC VI +TM1T
AC VI +547T

Figure 4


A
AC VI + GFP
AC VI + 547T
AC VI + GFP
GFP
- RT
AC VI
GAPDH
1kb

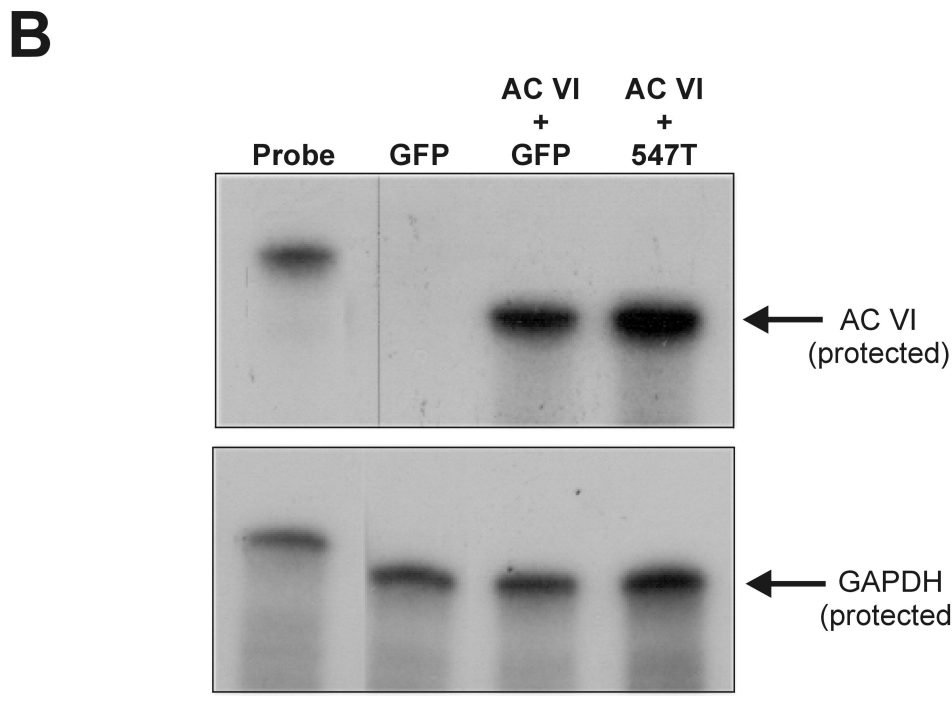

B
Probe
GFP
AC VI + GFP
AC VI + 547T
AC VI (protected)
GAPDH (protected)

Figure 5

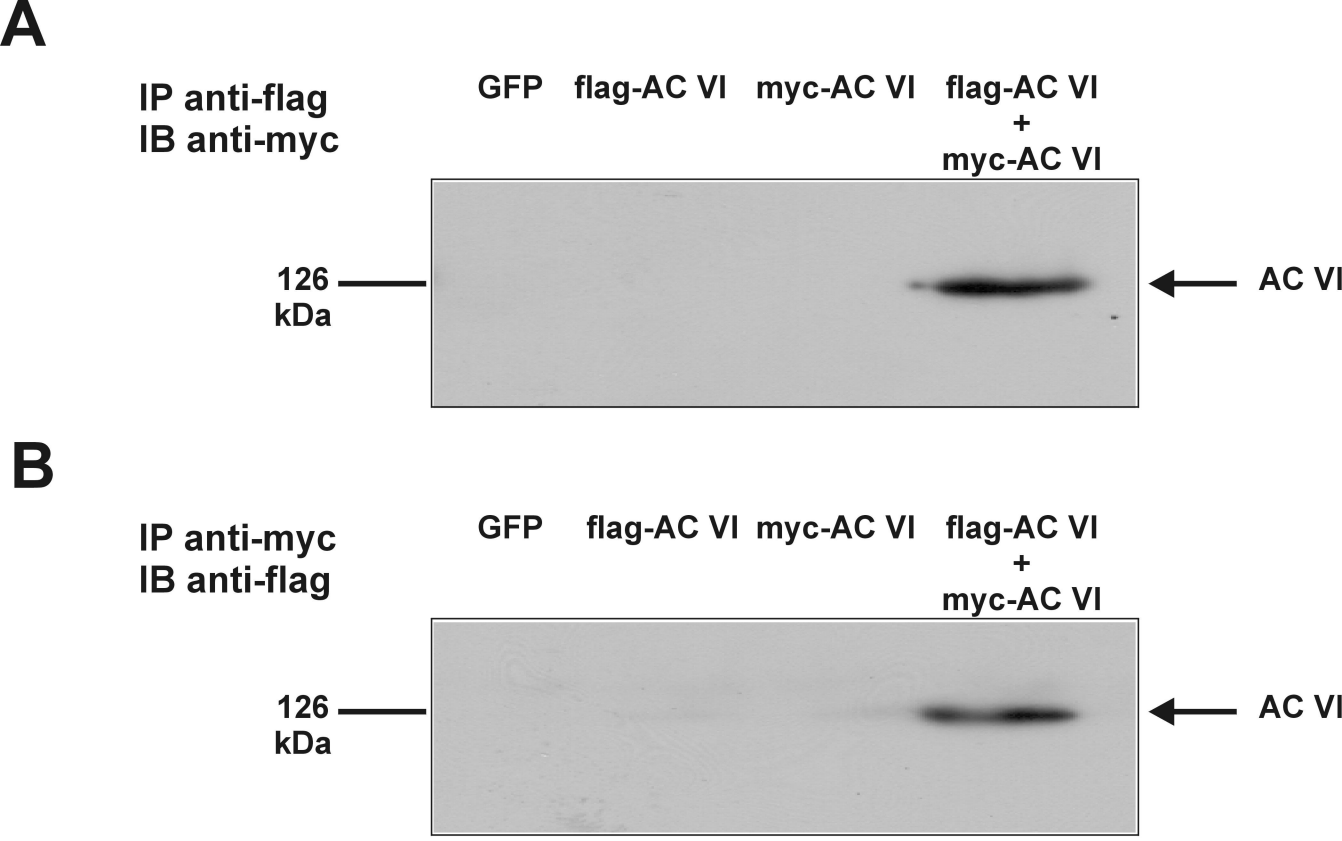

A
IP anti-flag
IB anti-myc
GFP
flag-AC VI
myc-AC VI
flag-AC VI
+
myc-AC VI
126
kDa
AC VI
B
IP anti-myc
IB anti-flag
GFP
flag-AC VI
myc-AC VI
flag-AC VI
+
myc-AC VI
126
kDa
AC VI

Figure 6

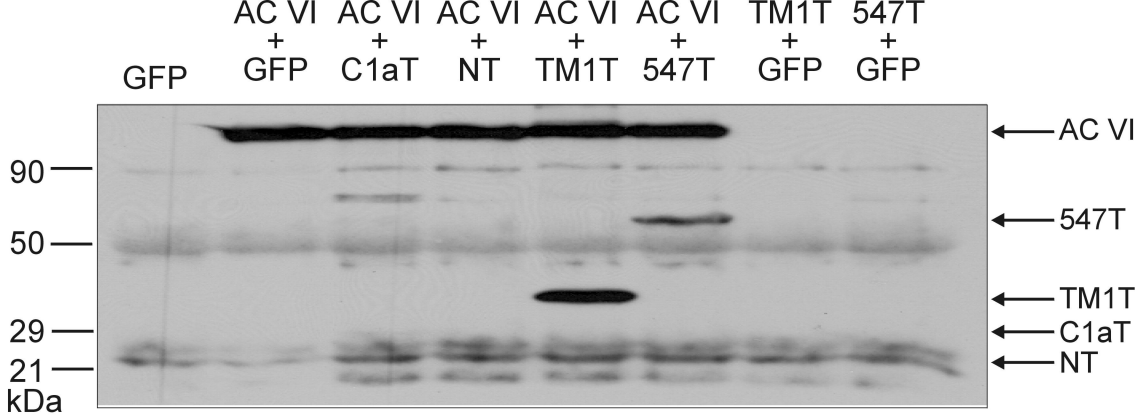

GFP
AC VI + GFP
AC VI + C1aT
AC VI + NT
AC VI + TM1T
AC VI + 547T
TM1T + GFP
547T + GFP
90
50
29
21
kDa
AC VI
547T
TM1T
C1aT
NT

Figure 7

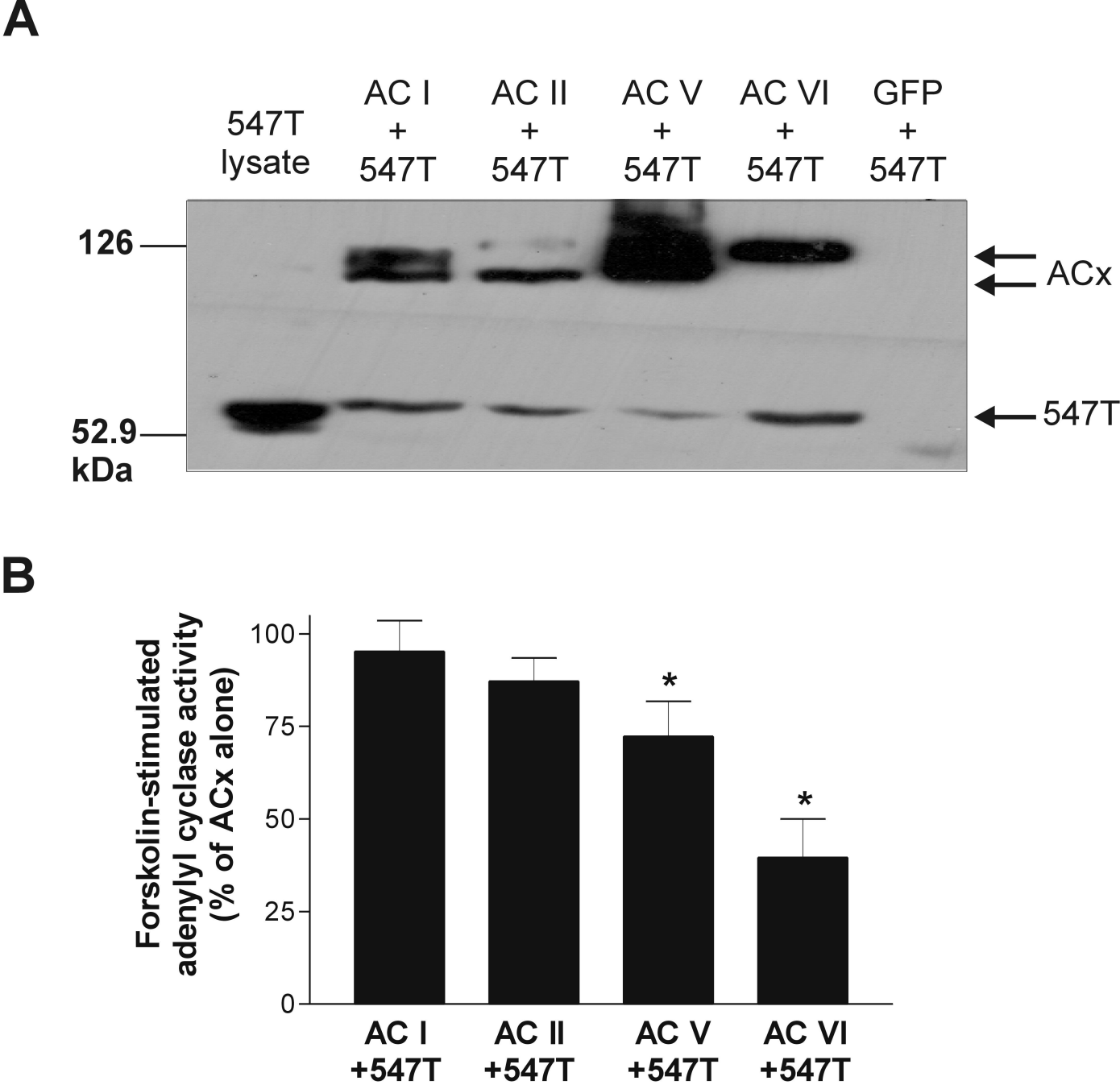

A
547T lysate
AC I + 547T
AC II + 547T
AC V + 547T
AC VI + 547T
GFP + 547T
126
52.9 kDa
ACx
547T
B
Forskolin-stimulated adenylyl cyclase activity (% of ACx alone)
100
75
50
25
0
*
*
AC I +547T
AC II +547T
AC V +547T
AC VI +547T

Figure 8