Histidine residues 912 and 913 in Protein Associated with Myc (PAM) are necessary for the inhibition of Adenylyl Cyclase Activity

Xianlong Gao and Tarun B. Patel†

Department of Pharmacology & Experimental Therapeutics
Loyola University Chicago
Stritch School of Medicine
2160 South First Avenue
Maywood, IL 60153
Tel: (708) 216-5773
Fax: (708) 216-6888
Email: tpatel7@lumc.edu

† Corresponding author.
RE: MOLPHARM/2004/005355

Running Title: H912 and H913 are critical for the inhibition of ACV by PAM

Corresponding Author: Tarun B. Patel

Department of Pharmacology & Experimental Therapeutics
Loyola University Chicago, Stritch School of Medicine
2160 S. First Avenue, Maywood, IL 60153
Tel: (708) 216-5773; Fax: (708) 216-6888
Email: tpatel7@lumc.edu

Text Statistics:
Number of Total Pages: 32
Words in Abstract: 244
Words in Introduction: 746
Words in Results and Discussion: 3135

Tables: 0
Figures: 7
References: 32

Abbreviations: AC, adenylyl cyclase; Roman numeral after AC represents isoform type of the enzyme; ACV-C2, C2 domain of type V adenylyl cyclase; G\( \alpha_s^* \), constitutively active (Q213L) mutant of the short splice variant of the \( \alpha \) subunit of the stimulatory GTP binding protein of adenylyl cyclase; cAMP, cyclic AMP; PAM, protein associated with c-Myc; for definition of PAM-N, RHD2, RHD2CT/22, and RHD2\( \Delta \)11 see Figure 1; DAPI, 4',6-Diamidino-2-phenylindole.
Abstract

Previously, we reported that Protein Associated with Myc (PAM) interacts with the C2 domain of type V adenylyl cyclase (ACV-C2) and that purified PAM is a potent inhibitor of Gαs- stimulated ACV activity (Scholich et al. J. Biol Chem. 276, 47583-47589, 2001). The present study was conducted to identify the region in PAM that inhibit ACV activity and to determine whether its binding with the ACV-C2 is necessary and sufficient to inhibit the enzyme. Co-expression of ACV and full-length PAM or its N-terminal third (PAM-N) in COS-7 cells inhibited isoproterenol- stimulated cAMP accumulation. Deletion of the RCC1 homology domains in PAM-N abolished its ability to inhibit isoproterenol-stimulated cAMP formation in cells. Purified GST fusion protein of the second RCC1 homology domain (RHD2) of PAM was sufficient to bind with ACV-C2 and inhibit Gαs- stimulated ACV activity. Additionally, deletion of 11 amino acids in GST-RHD2 obliterated its ability to bind with and inhibit ACV. The C-terminus of the RHD2 domain bound with ACV-C2 without inhibiting enzyme activity. Furthermore, substitution of H912 and H913 with alanine in the GST-RHD2 obliterated its ability to inhibit ACV without altering binding to ACV-C2. Likewise, H912/913A mutants of both PAM-N and full-length PAM did not inhibit cAMP formation in cells. Thus, the RHD2 domain of PAM is sufficient to inhibit Gαs- stimulated ACV activity and the binding of RHD2 to ACV-C2 is necessary but not sufficient for this inhibition. Moreover, H912 and H913 in PAM are critical for inhibiting ACV.
Introduction

Adenylyl cyclase (AC) catalyzes the conversion of ATP to the second messenger cyclic AMP which plays a crucial role in regulating a variety of physiological functions in response to hormones and neurotransmitters. Nine distinct and two splice variant forms of membrane-bound mammalian ACs have been cloned and characterized to date (reviewed in (Defer et al., 2000; Patel et al., 2001a; Smit and Iyengar, 1998)). All these enzymes are activated by GTP-bound α subunit of the stimulatory GTP-binding protein (Gαs) and, with the exception of ACIX, also by a synthetic diterpene, forskolin (see (Defer et al., 2000; Patel et al., 2001a; Smit and Iyengar, 1998; Tang and Gilman, 1992) for reviews). All of the membrane-bound isoforms of adenylyl cyclase share a characteristic structure, consisting of a short variable N terminus followed by two sets of six transmembrane spans that are separated by a large cytosolic domain (C1) (see (Defer et al., 2000; Patel et al., 2001a; Smit and Iyengar, 1998; Tang and Gilman, 1992) for reviews). The second set of transmembrane domains is followed by a cytoplasmic C-terminal domain (C2) (reviewed in (Defer et al., 2000; Patel et al., 2001a; Smit and Iyengar, 1998; Tang and Gilman, 1992)). The C1 and C2 domains of all isoforms share significant amino acid sequence homology and are sufficient to form a catalytic core with AC activity (Scholich et al., 1997a; Scholich et al., 1997b; Tang and Gilman, 1995; Wittpooth et al., 1999; Yan et al., 1996). Indeed, the separately expressed C1 and C2 domains, when mixed together, can reconstitute AC activity (Wittpooth et al., 1999; Yan et al., 1996). Both, the C1 and C2, domains also contribute to the regulation of AC by several modulators. For example, Gαi, bacterial cis-transpeptidylprolyl isomerase and RGS2 have been demonstrated to inhibit AC activity by interaction with C1 domain (Dessauer et al., 1998; Patel et al., 2001b; Sinnarajah et al., 2001; Wittpooth et al., 1999; Yan et al., 2001); forskolin and Gαs stimulate AC activity by interaction with both C1 and C2
domains (Scholich et al., 1997a; Scholich et al., 1997b; Sunahara et al., 1997; Tang and Gilman, 1995; Wittphoth et al., 1999; Yan et al., 1996; Yan et al., 1997).

In a previous report, using the yeast two-hybrid assay we identified a short portion of the protein associated with c-Myc (PAM) that interacts with the C2 domain of type V adenylyl cyclase (ACV) (Scholich et al., 2001). We also demonstrated that PAM purified from HeLa cells is a very potent inhibitor on some isoforms of AC, including AC1, ACV and ACs expressed in S49 cell membranes (Scholich et al., 2001). Additionally, we showed that a region of PAM comprising aa 446-1062 that contains the two regulator of chromosome condensation (RCC1)-homology domains (RHD1 and RHD2) was as potent as full-length PAM at inhibiting ACV activity (Scholich et al., 2001). In addition to inhibiting AC activity, PAM has also been shown to interact with c-Myc through a c-Myc binding domain (Guo et al., 1998). Mammalian PAM, by inhibiting adenylyl cyclase activity appears to play an important role in decreasing nociception (Ehnert et al., 2004). Furthermore, mammalian PAM by translocating to the plasma membrane may also provide the longer-term inhibition of adenylyl cyclase that is observed with sphingosine-1-phosphate (Pierre et al., 2004). These findings underscore the need to better understand the interactions between human PAM and adenylyl cyclases.

The RHD1 and RHD2 domains, but not the c-Myc-binding domain, are also present in the PAM homologues in Drosophila (HIW) and in C. elegans (RPM-1) (Schaefer et al., 2000; Wan et al., 2000; Zhen et al., 2000). These PAM homologues in Drosophila and C. elegans have been shown to be important in synaptogenesis at neuromuscular junctions (Schaefer et al., 2000; Wan et al., 2000; Zhen et al., 2000). Moreover, the RHD2 domain of RPM-1 appear to be crucial for its role in synaptogenesis since replacement of a histidine by alanine (H778A) within this domain failed to rescue the RPM-1 mutant phenotype (Zhen et al., 2000). This histidine
corresponds to H912 and H913 in human PAM. Here, we report that overexpression of full-
length PAM or its N-terminal one third (PAM-N) decreases the formation of cAMP in COS-7
cells stimulated by isoproterenol. The inhibitory effect of PAM is attributed to RHD2 domain.
Additionally, the C2-binding region in RHD2 is necessary but not sufficient for the inhibition of
AC activity. Furthermore, the H912/913A mutation in the RHD2 domain, PAM-N and full-
length PAM impairs the ability of these proteins to inhibit AC.
Materials and Methods

Plasmid Constructs: The 14 kb long full length of human PAM cDNA was pieced together from the cDNA fragments that were generated by RT-PCR using HeLa cell mRNA. The N-terminal fragment (nucleotides: 1-4512) was amplified with primers flanked by restriction sites Bam HI and Spe I (blunted) and was ligated into plasmid pCMV-Tag 1 at the Bgl II and EcoR V sites in order to gain the N-terminus FLAG tag and a C-terminus Myc epitope. This construct was then digested with Not I and Pvu I (Not I site blunted with Klenow) and cloned into the Pme I site of plasmid pcDNA 3.1. This generated the construct PAM-N that encodes for aa 1-1504 and has an N-terminal FLAG tag. The PCR product for nucleotides 4468-9300 with 3’ primer containing Xho I site was digested with Xba I and Xho I and inserted in the plasmid pcDNA 3.1 containing PAM-N. Finally, the PCR product corresponding to nucleotides 9191-13923 of PAM was generated using a 3’ primer that contained a Xho I site and this was then inserted in the pcDNA 3.1 construct described above at the Afl II and Xho I site. The full-length PAM cDNA was checked for sequence authenticity and in-frame cloning with the FLAG and Myc tags. For expression of proteins in bacteria (Escherichia coli), PAM fragments were synthesized by PCR and cloned between Bam HI and Xho I sites in the vector pGEX-4T-3, generating constructs expressing GST fusion proteins. The H912A, H913A, and H912/913A mutations as well as deletion of 11 residues (1042-1052) of PAM or its regions were created using Quickchange mutagenesis kit (Stratagene). All constructs were confirmed by sequencing. The full-length PAM and its derivative constructs used in our studies are shown in figure 1.

Immunocytochemistry: COS-7 cells (80,000 cells) were grown in Nunc tissue tech chambers and transfected with 0.5 µg each of the constructs PAM-N (Fig. 1) or PAM-NΔRCC1 using lipofectamine. One day later the medium was withdrawn and cells were fixed with 100%
ethanol for 10 min at -20 °C. This was followed by incubating the cells at room temperature for 1 min with a 1:1 mixture of methanol:acetone. After washing with PBS, the cells were permeabilized with 0.3% triton in PBS for five minutes and blocked with 10% normal goat serum in PBS containing Mg^{2+} and Ca^{2+} for 1 h at room temperature. Thereafter, the cells were incubated overnight at 4 °C with the monoclonal anti-FLAG antibody (M2 from Sigma Chemicals, Inc.; 1:250 dilution). Following three rinses (5 min each) with PBS, the cells were incubated with goat anti-mouse antibody conjugated with Alexa fluor 594 (Molecular Probes; 1:500 dilution with 10% goat serum in PBS). This step was followed by three rinses (5 min each) with PBS and slides were mounted with medium that contains 4′,6-Diamidino-2-phenylindole (DAPI) (Vector Labs, Inc.).

**Purification of bacterially expressed recombinant Gαs* and C1 or C2 domains of ACV:** Hexa-histidyl tagged constitutively active (Q213L) form of the short Gαs (Gαs*) was expressed in *E. coli* strain BL21(DE3) and purified as described in our previous publications (Scholich et al., 1997a; Wittphoth et al., 1999). As monitored by GTPγS binding, 20% of the Gαs* was active. The C1 and C2 domains of ACV were expressed in BL21(DE3) strain of *E.coli* as inclusion bodies. The inclusion bodies were extensively washed with buffer containing 10 mM Tris-HCl, pH 8.0 and 1mM EDTA. The resulting clean pellet was resuspended with 6M guanidine HCl and the solubilized proteins (20 ml, 1 mg/ml) were refolded at 4 °C by slow infusion (1.5 ml/hr) into 2 L of a solution containing 10mM Na₃PO₄, pH 7.4, 10 mM sodium pyrophosphate, 1 mM DTT, 0.1 mM MgCl₂, 0.1 mM MnCl₂, and 20% glycerol. Following centrifugation (20,000 g x 20 min), the refolded proteins were absorbed onto a HiTrap Q column and eluted with a gradient of 100-500 mM NaCl in 25 mM Tris-HCl, pH 7.4, 1 mM DTT, and 20% glycerol. The fractions containing C1 or C2 were identified by Coomassie blue staining, pooled, and the buffer was
exchanged using Centricon (Orbital Biosciences, QMWL: 10 kDa) with 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 50 mM NaCl, and 20% glycerol. The resulting purified C1 and C2 proteins when mixed together demonstrated significant adenylyl cyclase activity and were frozen at -80 °C.

**Purification of GST-fused PAM proteins:** GST tagged PAM proteins, GST-RHD2 (residues 861-1075), GST-RHD2-H912A, GST-RHD2-H913A, GST-RHD2-H912/913A, GST-RHD2Δ11, and GST-RHD2CT/22 were all expressed in *Escherichia coli* strain BL21. A schematic of these constructs is provided in figure 1. The expression was induced with 0.1 mM IPTG at 20 °C for 15 h. Bacteria were lysed by sonication in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 1 mM benzamidine, and 10 µg/ml each of aprotinin, leupeptin, and soybean trypsin inhibitor). After clarification by centrifugation, the lysate was supplemented with NaCl and Triton X-100 so that the final concentrations of these ingredients were 200 mM and 0.3% (v/v), respectively, before loading onto a glutathione affinity column. The column was washed twice with 10 volumes of lysis buffer containing 200 mM NaCl and 0.1% Triton X-100 and once with 10 volumes of lysis buffer containing 20 mM NaCl. Proteins were eluted with 50 mM Tris-HCl, 2 mM DTT, 20 mM NaCl and 10 mM glutathione. The entire eluate was directly applied to Mono Q 5/5 column and washed with the starting elution buffer. Proteins were eluted with a gradient of 0-500 mM NaCl in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM DTT. Fractions containing the proteins of interest were identified by Coomassie blue staining, pooled, and buffer was exchanged with 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT and 50 mM NaCl, and 10% glycerol using Centricon (Orbital Biosciences, QMWL: 10 kDa).

**Expression of ACV in Sf9 cells:** Sf9 cells were infected with recombinant baculovirus derived from ACV cDNA as described previously (Patel et al., 2001b; Scholich et al., 1997a). Sixty
hours after infection, the cells were harvested in PBS containing 1 mM benzanidine and 10 µg/ml each of aprotinin, leupeptin, and soybean trypsin inhibitor. The cells were lysed in 25 mM Hepes, pH 7.4, 1 mM EGTA, 10% sucrose with protease inhibitors and aliquots were stored at –80 °C until use.

**Adenylyl cyclase assay:** The adenylyl cyclase activity was assayed as described in our previous papers (Nair et al., 1989; Sun et al., 1995). The reaction was carried out in 100 µl in the presence of 5 mM of MgCl₂. Recombinant GST-PAM polypeptides or GST (control) were preincubated on ice for 20 min with membranes (10 µg protein) from Sf9 cells expressing ACV. This mixture was added to the assay reaction with either 50 nM of active Gαs* that had been activated with GTPγS or 100 µM of forskolin. AC activity was monitored over 15 min.

**Cyclic AMP formation in cells:** The method used was that of Salomon (Salomon, 1991). Essentially, COS-7 cells were cultured in 24-well plates in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum and 1% penicilin and streptomycin. Using Lipofectamine (Invitrogen), cells were co-transfected with 0.1 µg/well of pcDNA3-ACV together with 0.1 µg of plasmid encoding PAM fragments (for expression of full-length PAM, 0.15 µg of plasmid construct was used). Forty hours after transfection, cells were labeled with [³H]-adenine (1 µCi/well) for 4 h in DMEM without serum. Cells were then washed twice with DMEM and preincubated for 30 min with DMEM containing the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (0.2 mM) prior to the addition of agonist, isoproterenol or forskolin for 5 min at the concentrations indicated. Incubations were terminated by addition of 10% ice-cold trichloroacetic acid containing ¹⁴C labeled cAMP as an internal standard to correct for recovery. Cyclic-AMP in the trichloroacetic acid extract was isolated by using two sequential columns as described for adenylyl cyclase assay. The cAMP formation is calculated and expressed as a
percentage of conversion of total [3H]-adenine uptake in the cells. For each experiment, in parallel samples, the expression of FLAG-PAM, FLAG-PAM-N, FLAG-PAM-N-ΔRCC1, FLAG-PAM-N-H912/913A, and FLAG-PAM-H912/913A were determined by Western blotting using biotinylated anti-FLAG antibody (Sigma).

**Protein Binding Assay:** The binding assay was conducted *in vitro* using purified C1 or C2 domains of ACV along with pure GST-tagged PAM proteins. Clarified bacterial lysates containing GST-tagged PAM proteins were first incubated with glutathione resin and washed 3 times with 10 resin volumes of binding buffer (20mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, and 0.1% Triton X-100). The amount of GST-tagged protein bound to the resin was determined by SDS-PAGE and Coomassie staining using BSA as a standard. Ten microliters of the resin bound to GST tagged PAM proteins was added to 200 µl of binding buffer (described above) containing 0.15 µM each of either C1 or C2 and incubated for 1 h at 4 °C. After extensive washing with the binding buffer, the bound proteins were released from beads with Laemmli sample buffer and separated by SDS-PAGE. The anti-Xpress epitope tagged C1 or C2 were detected by immunoblotting with anti-Xpress antibody (Invitrogen).
Results and Discussion

In our previous report, using in-vitro assays, we demonstrated that PAM purified from HeLa cells inhibited Goαs-stimulated activity of type I (ACI) and type V (ACV) ACs as well as the ACs present in S49 cell membrane (ACVI and ACVII) (Scholich et al., 2001). Moreover, we also showed that a large region encompassed by aa 446-1062 of PAM was as potent an inhibitor of ACV as the full-length PAM (Scholich et al., 2001). In that study, using anti-sense oligodeoxynucleotide we showed that decreased expression of PAM in HeLa cells increased the ability of vasoactive intestinal peptide to increase cAMP accumulation (Scholich et al., 2001). However, this was not complemented by opposite findings in cells transfected to overexpress PAM because of the lack of a full-length (14 kb) clone of the PAM cDNA. Therefore, our initial approach was to construct the full-length PAM cDNA and to express the full-length protein along with ACV in COS-7 cells and to determine if the basal or agonist-stimulated cAMP formation was altered. ACV co-expression was necessary because transfection of PAM alone did not inhibit either forskolin- or isoproterenol- stimulated activity of endogenous adenylyl cyclases in COS-7 cells (data not shown). The lack of an effect of PAM on endogenous AC activity in COS-7 cells suggests that type VII and IX isoforms of AC that are expressed endogenously in COS-7 cells (Premont et al., 1996) are not inhibited by PAM. Because cAMP formation in the cells is monitored in the presence of the cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methyloxanthine, this assay actually measures intracellular AC activity (Salomon, 1991). As shown in figure 2A, the expression of full-length PAM together with ACV in COS-7 cells decreased the ability of isoproterenol (1 µM) to stimulate cAMP formation; in controls transfected with empty plasmid, the isoproterenol-stimulated cAMP accumulation was not altered (Fig. 2A). However, expression of the full-length PAM did not alter the ability of
RE: MOLPHARM/2004/005355

forskolin to stimulate cAMP formation in intact cells that were expressing ACV (Fig. 2A). Because the β-adrenoreceptor agonist isoproterenol stimulates AC activity in intact cells via activation of Goαs (reviewed in (Slotkin et al., 2003) (Gether et al., 2002)), these findings in intact cells (Fig. 2A) are in agreement with our previous in-vitro AC activity data (Scholich et al., 2001) in that PAM inhibits AC activity stimulated by Goαs but not by forskolin.

PAM is a large protein of 510 kD that contains a number of potentially functional domains (Guo et al., 1998). Since the N terminal fragment of PAM, PAM-N (residues 1-1504) contains the region (aa 446-1062, see Fig. 1) that we showed can inhibit ACV activity in-vitro (Scholich et al., 2001), we investigated whether PAM-N inhibited AC activity in intact cells. As shown in figure 2B, the dose-response curve of isoproterenol- stimulated cAMP formation in cells expressing PAM-N was shifted to the right. Thus, like the full-length PAM, PAM-N also inhibited cAMP formation in response to the β-adrenoreceptor agonist, isoproterenol. Notably, PAM-N also inhibited the ability of maximally effective concentrations of isoproterenol (10 µM) to inhibit cAMP accumulation (Fig. 2B, inset) indicating that the inhibition of cAMP formation by PAM-N is not reversed by high concentrations of isoproterenol. On the other hand, the ability of different concentrations of forskolin to stimulate AC activity in COS-7 cells was not altered by the expression of PAM-N (Fig. 2C). In these experiments, that were performed in parallel, by Western analyses with anti-FLAG antibody, we ensured that PAM-N was expressed to equivalent levels in cells treated with isoproterenol or forskolin (see representative blots shown in Figs. 2B and 2C). Previously, in in-vitro studies, we demonstrated that the full-length PAM and its RCC1-like domain (aa 446-1062) are equipotent at inhibiting ACV. However, from the data in figures 2A and 2B, it would appear that the full-length PAM is less potent than PAM-N at inhibiting ACV activity. This apparent discrepancy is explained by the fact that in the intact cell...
experiments, the expression of the full-length PAM (4641 amino acids) is always lower than that of the 1504 amino acid long PAM-N (not shown) and, therefore, the concentration of the full-length protein in cells may be lower. Thus, the data from experiments concerning expression of PAM-N and full-length PAM cannot be directly compared with each other. It should also be noted that expression of PAM or its N-terminal third (PAM-N) does not alter the expression of ACV since the forskolin stimulated activity was the same whether or not PAM or its derivative, PAM-N were expressed (Figs. 2A and 2C).

PAM contains two regions, RHD1 (aa 498-740) and RHD2 (aa 874-1065), that are similar to two parts of the regulator of chromosome condensation 1 (RCC1). RCC1 is a guanine nucleotide exchange factor for the small G protein, Ran (Bischoff and Ponstingl, 1991; Carazo-Salas et al., 1999) and its structure, a seven bladed propeller, is similar to the β subunit of heterotrimeric G proteins (Sondek et al., 1996). Previously, in in-vitro assays, we demonstrated that a protein corresponding to the region of PAM that contained both the RHD1 and RHD2 domains could inhibit ACV activity in-vitro (Scholich et al., 2001). Therefore, to determine if the RCC1-like region of PAM is necessary for inhibition of AC activity in intact cells, we transfected cells with PAM-N or its deletion mutant lacking the RHD1 and RHD2 regions (PAM-NΔRCC1). As shown in figure 3, although PAM-N and PAM-NΔRCC1 were expressed to the same level (Fig. 3A), the expression of PAM-N, but not PAM-NΔRCC1, inhibited the ability of sub-maximal concentration of isoproterenol to stimulate cAMP formation in COS-7 cells. In parallel experiments, neither PAM-N nor PAM-NΔRCC1 altered the ability of forskolin to stimulate cAMP accumulation in cells (Fig. 3C). These findings are consistent with our previous in-vitro experiments (Scholich et al., 2001) which showed that the RCC1-like region of PAM is necessary to observe the inhibition of isoproterenol- stimulated AC activity in intact
cells. That the lack of inhibition of ACV by PAM-NΔRCC1 is not due to ectopic localization of the protein in some compartment of the cells is shown by the fact that both PAM-N and PAM-NΔRCC1 were distributed similarly in cells (Fig. 3D). In these experiments (Fig. 3D), specificity of the anti-FLAG antibody for the FLAG-tagged proteins is shown by the fact that the untransfected cells showed nuclear staining alone (Fig. 3D) indicating that the anti-FLAG antibody does not recognize cellular proteins in a non-specific manner. The lack of inhibition of ACV by PAM-NΔRCC1 (Fig. 3B) cannot be explained by alterations in the expression of ACV since the forskolin- stimulated activities in cells transfected with PAM-N and PAM-NΔRCC1 were similar (Fig. 3C).

Within the RCC1-like region, the RHD1 and RHD2 domains correspond to the first four and the last three β-propeller blades of RCC1, respectively. In the yeast two-hybrid screen that we performed with the C2 domain of ACV (ACV-C2) as bait, the positive clones contained the cDNA corresponding to aa 1028-1231 of PAM (Scholich et al., 2001). This latter region contains the C-terminal 38 amino acids (aa 1028-1065) of the RHD2 domain that form part of the seventh propeller blade (see Fig. 1). Therefore, the next series of experiments were performed to address the hypothesis that the RHD2 domain of PAM, or a part thereof, was necessary for interactions with ACV-C2 and/or inhibition of AC activity.

To determine whether the RHD2 domain of PAM could inhibit ACV activity, the ability of the purified GST- fusion protein containing the RHD2 region and its flanking amino acids (aa 861-1075 of PAM, Fig. 1) to inhibit ACV activity was monitored. For this purpose, membranes of Sf9 cells infected to express ACV were used. The forskolin- or Gαs- stimulated endogenous AC activity in Sf9 cell membranes was not inhibited by GST-RHD2 (data not shown). As shown in Figure 4A, GST-RHD2, but not GST alone, in a concentration-dependent manner,
inhibited the Gαs*- stimulated ACV activity in Sf9 cell membranes. As expected from our previous findings (Figs. 1 & 2, and (Scholich et al., 2001)) neither GST-RHD2 nor GST alone inhibited the forskolin- stimulated activity of ACV. Interestingly, as compared with the RCC1-like domain (aa 446-1062) (16), the RHD2 domain (aa 861-1075) was less potent at inhibiting ACV activity. These data suggest that additional residues N-terminus of the RHD2 domain are involved in increasing the potency of the inhibition. Previously, we and others have shown that together, Gαs and forskolin can stimulate the activity of ACV to a greater level than either agent alone (Scholich et al., 1997a). Thus, we investigated if the RHD2 domain inhibited the activity of ACV that was maximally stimulated with a combination of forskolin and Gαs. As shown in figure 4B, when Gαs and forskolin were present together, the activity of ACV was stimulated to a greater extent than either agent alone. While the forskolin-stimulated activity of ACV was not inhibited, the GST-RHD2 inhibited the Gαs plus forskolin- stimulated ACV activity (Fig. 4B). These data suggest that the GST-RHD2 inhibits Gαs- stimulated component of the activity that is maximally stimulated by forskolin plus Gαs.

Next we investigated whether the RHD2 domain interacted with the C1 or C2 regions of ACV. In these experiments, the ability of GST or GST-RHD2 to bind the purified C1 and C2 domains of ACV was investigated. Figure 5A shows that the RHD2 domain interacts with ACV-C2 but not the C1 domain of the enzyme; GST alone (control) did not interact with either of the two ACV domains (Fig. 5A). As mentioned above, the N-terminus of the clone that interacted with ACV-C2 in the yeast two-hybrid assay (aa 1028-1231, (Scholich et al., 2001)) has 38 amino acids that overlap with the C-terminus of the RHD2 domain of PAM (Fig. 1). Because both these proteins bind ACV-C2 (Fig. 5A and (Scholich et al., 2001)), we reasoned that these overlapping 38 amino acids form the ACV-C2 binding region on PAM. Therefore, to address
the question of whether the binding of GST-RHD2 to ACV-C2 was necessary for inhibition of ACV activity, we deleted 11 amino acids (aa 1042-1052) in the middle of the 38 aa overlapping region in the construct GST-RHD2 to generate the protein GST-RHD2Δ11 (Fig. 1). Indeed, as shown in figure 5A, GST-RHD2Δ11 did not bind with ACV-C2 indicating that these amino acids reside in the ACV-C2 binding region of PAM. Moreover, the ability of GST-RHD2Δ11 to inhibit Gαs*-stimulated ACV activity was markedly decreased; significant inhibition was only observed at the maximal concentration (300 nM) tested (Fig. 5B). In contrast, the GST-RHD2 inhibited Gαs*-stimulated activity with an EC₅₀ of approximately 10 nM. These findings (Fig. 5) demonstrate that the binding of the GST-RHD2 to ACV-C2 is necessary for inhibition of ACV activity.

To determine whether the binding of a region of PAM to ACV-C2 is by itself sufficient to inhibit enzyme activity, we made another GST-fusion protein comprising of aa 977-1231 of PAM. This construct (GST-RHD2CT/22) contains the entire region (aa 1028-1231) encoded by the clone that interacted with ACV-C2 in the yeast two-hybrid assay (Scholich et al., 2001) plus 52 additional amino acids in the C-terminus of the RHD2 domain (Fig. 1). As expected, GST-RHD2CT/22 bound ACV-C2, but not the C1 region of ACV (Fig. 5A). However, as compared with GST-RHD2, the ability of GST-RHD2CT/22 to inhibit Gαs*-stimulated ACV activity was markedly diminished (Fig. 5B). These latter findings suggest that the binding of the C terminus of RHD2 to the ACV-C2 domain is not sufficient to observe inhibition of ACV activity and that the N terminal part of RHD2 is necessary for this effect.

The PAM homologues in C. elegans (RPM-1) and in Drosophila (HIW) contain the RHD regions (Schaefer et al., 2000; Wan et al., 2000; Zhen et al., 2000). In C. elegans, RPM-1 mutant exhibited a phenotype that resulted in defective synaptogenesis and neuromuscular junction
formation (Zhen et al., 2000). This defect could be rescued by wild type RPM1 but not its mutant carrying a single H778A substitution, suggesting the crucial role of the histidine residue (Zhen et al., 2000). This histidine residue is located in the RHD2 domain of RPM-1 (Zhen et al., 2000). Interestingly, human PAM contains two adjacent histidines (H912 and H913) that located within its RHD2 domain and correspond to H778 of RPM1 (Fig. 1). Therefore, we investigated whether either or both of these histidine residues in human PAM play any role in the inhibition of AC activity. Initially, we made single and double mutants of GST-RHD2 and examined their ability to inhibit Goαs*- stimulated ACV activity. As shown in figure 6A, when H912 or H913 were individually mutated to alanine, the ability of the RHD2 domain to inhibit ACV activity was attenuated by approximately 50%. However, the substitution of both H912 and H913 with alanine obliterated the ability of RHD2 domain of PAM to inhibit ACV (Fig. 6A). These findings demonstrate that both H912 and H913 play an essential role in inhibition of Goαs-stimulated ACV activity. Notably, mutation of histidines 912 and 913 to alanine in the GST-RHD2 (GST-RHD2-H912/913A) did not alter its binding to ACV-C2 (Fig. 6B) and did not inhibit ACV activity even at high concentrations (Fig. 6B, lower panel). These data confirm the notion that binding of RHD2 to ACV-C2 is not sufficient to inhibit ACV activity. Moreover, the data in figure 6 demonstrate that the H912/913 in PAM are critical in mediating the inhibition of ACV activity and confirm the contention that the N-terminus of the RHD2 domain of PAM is necessary for inhibition of AC activity.

To examine the importance of the H912 and H913 residues in the RHD2 region in the context of a larger protein, the experiments shown in figure 7 were performed. Essentially, COS-7 cells were transfected with PAM-N, or full-length PAM and their point mutants, PAM-N-H912/913A and PAM-H912/913A. The expression of the proteins and their mutants to equal
levels was confirmed by Western analyses (Fig. 7, top panel) and the ability of isoproterenol or forskolin to stimulate cAMP formation was determined. As observed with the RHD2 domains in ACV activity assays (Fig. 6), PAM-N and PAM, but not PAM-N-H912/913A or PAM-H912/913A, inhibited the ability of isoproterenol to increase cAMP accumulation in intact cells (Fig. 7). As expected neither protein altered the ability of forskolin to stimulate cAMP accumulation. The finding that mutations of H912 and H913 in the small RHD2 construct as well as the longer PAM-N, or full-length PAM constructs obliterated the ability of the proteins to inhibit AC activity confirms that these amino acid play a pivotal role in modulating Gαs-stimulated AC activity.

The precise mechanisms by which PAM or its RHD2 domain decrease the ability of Gαs* to stimulate AC activity is presently unclear. However, since we used the constitutively active mutant of Gαs (Q213L, Gαs*) in our assays, it is unlikely that PAM or its RHD2 domain act as a GTPase activating protein to decrease the ability of Gαs* to activate AC activity. Consistent with this notion is our earlier observation that Gαs*- stimulated activity of type II AC is not inhibited by PAM (Scholich et al., 2001). It is also not likely that interaction of PAM with the C2 domain of ACV interferes with the interactions of Gαs* with this region of ACV since the mutation of H912/913 in the RHD2 domain that does not alter its binding to C2 is ineffective at altering the ability of Gαs* to stimulate AC activity. These data also suggest that the binding of PAM to C2 domain of ACV per se does not alter catalytic activity of the enzyme and that the region N-terminus to the binding domain on PAM in some manner inhibits Gαs- stimulated activity. Because PAM inhibits the isoforms of AC that are inhibited by Gαi (ACV, ACVI and ACI), it is tempting to speculate that the protein binds the C2 domain and, that akin to the mechanism of Gαi inhibition, its N-terminus somehow alters the interactions between the C1 and
RE: MOLPHARM/2004/005355

C2 domains to inhibit Gαs- stimulated activity. However, this mode of inhibition has to be different to that of Gαi since forskolin-stimulated activity is not inhibited by PAM. Whatever, the mechanism, it is clear that the histidine residues (aa 912 and 913) in PAM play a critical role in the inhibition and further analyses will be necessary to define the precise manner by which this inhibition occurs.

Since PAM is a large protein that contains many protein homology domains including the c-Myc binding region (Guo et al., 1998), it may have multiple physiological functions. However, other than modulating AC activity and playing a role in nociception and long-term attenuation of AC activity by sphingosine-1-phosphate (Ehnert et al., 2004; Pierre et al., 2004) the other functions of mammalian PAM remains to be elucidated. A clue to the additional functions of mammalian PAM comes from its Drosophila and C. elegans homologues RPM-1 and HIW which have been found to regulate synaptogenesis and neuromuscular junction formation (Schaefer et al., 2000; Wan et al., 2000; Zhen et al., 2000). To this end, our previous findings that PAM is distributed in certain areas of the mammalian brain and that its distribution changes with development (Yang et al., 2002) coupled with the fact that PAM also inhibits ACI (16), a neuronal AC isoform, suggests that in mammals PAM may also be important in synaptogenesis. Our observation that mutation of the histidine residues in human PAM (PAM-N) which correspond to the histidine in RPM1 that is necessary to rescue function in the RPM-1 mutant of C. elegans, obliterates the ability of the protein to inhibit AC, tempts us to speculate that PAM may modulate synaptogenesis by regulating Gαs-stimulated AC activity. The isoforms of AC present in synaptic terminals at neuromuscular junctions are, presently, not known. However, it should be noted that in addition to ACV, PAM also inhibits ACI and ACVI (Scholich et al., 2001). In this respect the findings presented here may be applicable to several other isoforms of
RE: MOLPHARM/2004/005355

AC isoforms and the possibility that PAM regulates synaptogenesis at neuromuscular junctions by inhibiting AC activity needs to be formally addressed.

In conclusion, we have demonstrated that full-length PAM and its N-terminal third (PAM-N) can inhibit AC activity in intact cells. Using GST-fusion proteins representing smaller portions of PAM, we have demonstrated that the RHD2 domain of PAM is sufficient to inhibit ACV activity and that binding of this region to the C2 domain of ACV is necessary but not sufficient for inhibition of AC activity. Moreover, we have shown that in the context of both the short RHD2 domain and the larger PAM-N as well as full-length PAM proteins, the mutation of a histidine residues (H912 and H913) obliterates the ability of PAM to inhibit AC activity \textit{in vitro} as well as in intact cells. Future studies will investigate the mechanisms by which PAM and its RHD domain inhibit the ability of G\textsubscript{os} to stimulate AC activity.
Acknowledgments

We thank Dr. A. G. Gilman, Univ. of Texas Southwestern Medical School for providing us with the Gαs cDNA. We are also grateful to Dr. Yoshihiro Ishikawa, Univ. of Medicine and Dentistry of New Jersey, for the gift of the canine cDNAs encoding ACV.
References


RE: MOLPHARM/2004/005355

Footnotes

This research was supported by NIH grant HL59679 and a postdoctoral fellowship from the AHA, Midwest Consortium to XG.
Legends for Figures

Figure 1: Schematic representation of the full-length PAM and other PAM constructs used in the present study. The numbers denote the amino acid residues in human PAM. The C2 interacting clone refers to the partial clone of PAM that was isolated in our previous study (Scholich et al., 2001).

Figure 2: Full-length PAM and its N-terminus (PAM-N, aa 1-1504) inhibit cAMP accumulation stimulated by isoproterenol but not by forskolin. COS-7 cells (4 x 10^4 cells/well in 24 well plate) were co-transfected with plasmid expressing ACV along with empty vector or constructs to express the FLAG-tagged full-length PAM (Panel A) or its N-terminus (PAM-N) (Panels B and C) in plasmid pcDNA3.1. Forty hours after transfection, cells were serum-starved and labeled with 1 μCi/well ^3H-adenine in DMEM for 4 h, followed by 30 min of incubation with 3-isobutyl-1-methylxanthine (0.2 mM). Cyclic AMP formation was stimulated with either 1 μM isoproterenol (Panel B), 10 μM isoproterenol (inset in Panel B) or various concentrations of the β-adrenoreceptor agonist (Panel B) for 5 minutes. Similarly, cells were treated with either 100 μM (Panel A) or varying concentrations of forskolin (Panel C) for 5 min. Cyclic AMP formation was determined as described under “Materials and Methods”. Expression of full-length PAM and PAM-N were monitored by Western analyses using anti-FLAG antibody (insets in Panels A-C). The data are presented as the mean ± S.E. and are representative of three experiments each performed in triplicate. *p < 0.05; ** p < 0.01, Student’s unpaired t-test.

Figure 3: Deletion of RCC1-like domains (498-1065) in PAM-N abolishes its ability to inhibit isoproterenol-stimulated cAMP accumulation. COS-7 cells (4 x 10^4 cells/well in 24 well plate) were transfected with constructs expressing ACV along with either empty vector,
pcDNA3.1-FLAG-PAM-N or pcDNA3.1-FLAG-PAM-NΔRCC1 as indicated. Expression of the proteins in cells was determined by Western Analyses with anti-FLAG antibody (Panel A). Cyclic AMP formation was stimulated with 1 μM isoproterenol (ISO, Panel B) or 100 μM forskolin (Fsk, Panel C) for 5 min and monitored as described under “Materials and Methods”. The data are presented as the means ± S.E. and are representative of at least three experiments each performed in triplicate. * p < 0.01, compared with vector + ISO, Student’s unpaired t-test. Panel D: PAM-N and PAM-NΔRCC1 were transfected into COS-7 cells and their intracellular localization was monitored using anti-FLAG antibody assay described in Materials and Methods section. The images shown are 400x magnification. PAM-N and PAM-NΔRCC1 are shown by Alexa fluor 594 (red) and nuclei are shown by DAPI staining (blue). Note the absence of both proteins in nucleus and the similar distribution. Also note that only cells expressing the FLAG-tagged proteins showed the red color (untransfected cells are seen by nuclear staining only) indicating that the staining with the anti-FLAG antibody is specific.

**Figure 4:** RHD2 domain of PAM inhibits Gαs*--, but not forskolin-, stimulated ACV activity. Amino acid residues (861-1065) encompassing the RHD2 domain of PAM was expressed as a GST fusion protein and purified from *E. coli* as described under Materials and Methods. The effect of different concentrations of GST or GST-RHD2 on AC activity in 10 µg of Sf9 membrane expressing ACV was examined. Panel A: The GST and GST-RHD2 were preincubated with the membranes and then assayed for AC activity in the presence of 50 nM of GTPγS-activated Gαs* or 100 μM forskolin for 15 min at room temperature. Adenylyl cyclase activities were determined as described under “Materials and Methods”. Control (100%) activity in the presence of Gαs or forskolin alone were
377.5 ± 32.2 pmol/min/mg and 378.8 ± 15.6 pmol/min/mg, respectively. Data presented are percent of these control activities ± S.E. of three experiments each performed in triplicate. *p<0.05, **p<0.01 as compared to control in the absence of GST-RHD2. 

**Panel B:** Same as panel A, except that the ACV activities were monitored at 100 nM GST-RHD2 and included experiments with both Gαs* (50 nM) plus forskolin (100 µM). Data shown are the mean ± S. E. (n=3). **p<01 as compared to control without GST-RHD2.

**Figure 5:** The binding of the RHD2 domain of PAM to the C2 region of ACV is required but not sufficient for its inhibitory effect on ACV. **Panel A:** Purified C1 and C2 domains (0.15 µM each) of ACV containing an N-terminal Xpress tag were incubated with GST, GST-RHD2 (residues 861-1075), GST-RHD2∆11, and GST-RHD2-CT/22 (residues 977-1231) proteins as described in Materials and Methods. The numbers in the figure indicate the concentrations of GST fusion proteins (nM) in the binding mixture. After incubation for 1 h at 4 °C and extensive washing, the bound C1 and C2 proteins were separated by SDS-PAGE and detected by immunoblotting using anti-Xpress antibody. **Panel B:** The effect of various indicated concentrations of GST and GST fusion proteins derived from PAM on AC activity in membranes of Sf9 cells expressing ACV was monitored. Membranes of Sf9 cells expressing ACV were preincubated with the proteins at the indicated concentrations and then assayed in the presence of 50 nM of Gαs* for 15 min at room temperature. Control (100%) activity in the presence of Gαs* alone was 428 ± 19.1 pmol/min/mg. Data are presented as percent of this control ± S.E. of three experiments each performed in triplicate. *p<0.05; **p<0.01; Students unpaired t-test.

**Figure 6:** Mutation of H912 and H913 to alanine in the RHD2 domain obliterates its ability to inhibit ACV activity but not its binding with the C2 domain of ACV. **Panel A:** H912 and
H913 in the RHD2 domain were mutated individually or together to alanine and the ability of 100 nM each of the wild-type RHD2 or its mutants to inhibit Gαs*-stimulated ACV activity was monitored as described in legend to figure 5. Data are the mean ± SEM and significance of the differences are shown (n = 6). #p<0.01 as compared with GST alone, † not significant as compared with GST alone, **p<0.01 between conditions shown by bars.

Panel B: The binding of different concentrations (in nM) of GST, GST-RHD2 or its mutant GST-RHD2-H912/913A to 0.15 µM C2 domain of ACV was monitored as described in legend to figure 5 and in “Materials and Methods”. Following SDS-PAGE, the bound C2 was monitored by Western analyses with anti-Xpress antibody. The ability of different concentrations of GST-RHD2 or GST-RHD2-H912/913A to inhibit Gαs* (50 nM) stimulated ACV activity in 10 µg of Sf9 membrane was also monitored as described in legend to figure 5 and under “Materials and Methods”. Data presented are the mean ± SEM of three experiments performed in triplicates. *p<0.05; **p<0.01, Student’s unpaired t-test.

Figure 7: The H912/913A mutations in PAM-N (the N terminus of PAM) and full-length PAM obliterate their ability to inhibit cAMP formation in intact cells. COS-7 cells were transfected with constructs expressing ACV and either pcDNA3.1 (vector), or pcDNA3.1 constructs expressing wild type PAM-N and full-length PAM or their mutants PAM-N-H912/913A and PAM-H912/913A as indicated. Expression of these proteins was monitored by Western analyses with anti-FLAG antibody (top panel). Cells were stimulated with 1 µM isoproterenol (ISO) or 100 µM forskolin (Fsk) for 5 min and cAMP production was measured as described under legends to figures 1 and 2 and in “Materials and Methods”. The data presented are the means ± S.E. of either three experiments each
performed in triplicate (panel B) or quadruplicates from a representative experiment (panel C). **p < 0.01, Student’s unpaired t-test.
Figure 5

(A) Western blot analysis showing the interaction of GST, GST-RHD2, GST-RHD2Δ11, GST-RHD2-CT/22 with C1 and C2 samples.

(B) Graph depicting the AC activity (% of control) as a function of protein concentration (nM). The graph compares GST, GST-RHD2, GST-RHD2Δ11, and GST-RHD2-CT/22. Significant differences are indicated by asterisks: * for p < 0.05 and ** for p < 0.01.