Histidine Residues 912 and 913 in Protein Associated with Myc Are Necessary for the Inhibition of Adenylyl Cyclase Activity

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ABSTRACT

We reported previously that protein associated with Myc (PAM) interacts with the C2 domain of type V adenyl cyclase (ACV-C2) and that purified PAM is a potent inhibitor of Gαs-stimulated ACV activity (J Biol Chem 276:47583–47589, 2001). The present study was conducted to identify the region in PAM that inhibits ACV activity and to determine whether its binding with the ACV-C2 is necessary and sufficient to inhibit the enzyme. Coexpression 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. In addition, 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 His912 and His913 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, His912 and His913 in PAM are critical for inhibiting ACV.
activity by interaction with both C1 and C2 domains (Tang and Gilman, 1995; Yan et al., 1996; Scholich et al., 1997a,b; Sunahara et al., 1997; Yan et al., 1997; Witttpoth et al., 1999).

In a previous study, we identified, using the yeast two-hybrid assay, a short portion of the protein associated with c-Myc (PAM) that interacts with the C2 domain of type V adenyl 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 ACI, ACV, and ACs expressed in S49 cell membranes (Scholich et al., 2001). In addition, 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 adenyl cyclase activity, seems to play an important role in decreasing nociception (Ehnhert et al., 2004). Furthermore, by translocating to the plasma membrane, mammalian PAM may also provide the longer term inhibition of adenyl 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 adenyl cyclases.

The RHD1 and RHD2 domains, but not the c-Myc-binding domain, are also present in the PAM homologues in Drosophila melanogaster (HIW) and in Caenorhabditis elegans (RPM-1) (Schaefe et al., 2000; Wan et al., 2000; Zhen et al., 2000). These PAM homologues in D. melanogaster and C. elegans have been shown to be important in synaptogenesis at neuromuscular junctions (Schaefe et al., 2000; Wan et al., 2000; Zhen et al., 2000). Moreover, the RHD2 domain of RPM-1 seems to be crucial for its role in synaptogenesis because replacement of a histidine with an alanine (H778A) within this domain failed to rescue the RPM-1 mutant phenotype (Zhen et al., 2000). This histidine corresponds to His912 and His913 in human PAM. Herein, we report that overexpression of full-length PAM or its N-terminal 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. In addition, 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 full length of human PAM cDNA was pieced together from the cDNA fragments that were generated by reverse transcription-PCR using HeLa cell mRNA. The N-terminal fragment (nucleotides 1–4512) was amplified with primers flanked by restriction sites BamHI and SpeI (blunted) and was ligated into plasmid pCMV-Tag1 at the BglIII and EcoRI sites to gain the N-terminal FLAG tag and a C-terminal Myc epitope. This construct was then digested with NotI and PvuI (NotI site blunted) and cloned into the pNel site of plasmid pcDNA 3.1. This generated the construct PAM-N that encodes for aa 1 to 1504 and has an N-terminal FLAG tag. The PCR product for nucleotides 4468 to 9300 with 3' primer containing XhoI site was digested with XhoI and XhoI and inserted in the plasmid pcDNA 3.1 containing PAM-N. Finally, the PCR product corresponding to nucleotides 9191 to 13,923 of PAM was generated using a 3' primer that contained a XhoI site and this was then inserted in the pcDNA 3.1 construct described above at the AffIII and XhoI 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 BamHI and XhoI 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 the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were confirmed by sequencing. The full-length PAM and its derivative constructs used in our studies are shown in Fig. 1.

Immunocytochemistry. COS-7 cells (80,000 cells) were grown in tissue tech chambers (Nalge Nunc International, Naperville, IL) and transfected with 0.5 µg of each of the constructs PAM-N (Fig. 1) or PAM-NΔRCC1 using LipofectAMINE. The next day, the medium was withdrawn and cells were fixed with 10% 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/acetic acid. After washing with PBS, the cells were permeabilized with 0.3% Triton in PBS for five min and blocked with 10% normal goat serum in PBS containing Mg2+ and Ca2+ 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, St. Louis, MO; 1:250 dilution). After three rinses (5 min each) with PBS, the cells were incubated with goat anti-mouse antibody conjugated with Alexa fluor 594 (1:500 dilution with 10% goat serum in PBS; Molecular Probes, Eugene, OR). 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 (Vector Labs, Inc., Burlingame, CA).

Purification of Bacterially Expressed Recombinant Gass and C1 or C2 Domains of ACV. The His6-tagged constitutively active (Q213L) form of the short Gass (Gass*) was expressed in E. coli strain BL21(DE3) and purified as we described previously (Scholich et al., 1997a; Witttpoth et al., 1999). As monitored by guanosine 5'-O-(3-[35S]thio)triphosphate binding, 20% of the Gass* 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 1 mM EDTA. The resulting clean pellet was resuspended with 6 M guanidine HCl and the solubilized proteins (20 ml, 1 mg/ml) were refolded...
at 4°C by slow infusion (1.5 ml/h) into 2 liters of a solution containing 10 mM NaPO4, pH 7.4, 10 mM sodium pyrophosphate, 1 mM DTT, 0.1 mM MgCl2, 0.1 mM MnCl2, and 20% glycerol. After centrifugation (20,000 g for 20 min), the reloaded proteins were absorbed onto a HiTrap Q column and eluted with a gradient of 100 to 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 and pooled, and the buffer was exchanged using Centriprep (quantitative molecular weight limit, 10 kDa; Orbital Biosciences, Topsfield, MA) 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 adenyl 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-RHD2CT2Δ2 were all expressed in Escherichia coli strain BL21. A schematic of these constructs is provided in Fig. 1. The expression was induced with 0.1 mM isopropyl-β-D-thiogalactoside 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 phenylmethylsulfonyl fluoride, 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 to 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 washed with 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT and 50 mM NaCl, and 10% glycerol using Centriprep (quantitative molecular weight limit, 10 kDa; Orbital Biosciences).

Adenylyl Cyclase Assay. The adenylyl cyclase activity was assayed as we have described previously (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 of protein) from Sf9 cells expressing ACV. This mixture was added to the assay reaction with either 50 nM of active Gaβ5 that had been activated with guanosine 5’-O-(3-thio)triphosphate or 100 μM of forskolin. AC activity was measured over 15 min.

Cyclic AMP Formation In Cells. The method used was that of Salomon (1991). COS-7 cells were cultured in 24-well plates in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum and 1% penicillin and streptomycin. Using LipofectAMINE (Invitrogen), cells were cotransfected with 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-methyloxanthine (0.2 mM) before the addition of agonist, isoproterenol, or forskolin for 5 min at the concentrations indicated in figures or figure legends. Incubations were terminated by addition of 10% ice-cold trichloroacetic acid containing [14C]cAMP as an internal standard to correct for recovery. cAMP in the trichloroacetic acid extract was isolated by using two sequential columns as described for adenyl cyclase activity. The cAMP formation was 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-ΔRC1, FLAG-PAM-N-H912/913A, and FLAG-PAM-H912/913A were determined by Western blotting using biotinylated anti-FLAG antibody (Sigma).

Results and Discussion

In our previous report, using in vitro assays, we demonstrated that PAM purified from HeLa cells inhibited Gαs-stimulated activity of AC1 and ACV 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 to 1062 of PAM was as potent an inhibitor of ACV as the full-length PAM (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, to express the full-length protein along with ACV in COS-7 cells, and to determine whether the basal or agonist-stimulated cAMP formation was altered. ACV expression was necessary because transfection of PAM alone inhibited neither forskolin- nor 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 Fig. 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 forskolin to stimulate cAMP formation in intact
cells that were expressing ACV (Fig. 2A). Because the β-adrenoceptor agonist isoproterenol stimulates AC activity in intact cells via activation of Gαs (for review, see Gether et al., 2002; Slotkin et al., 2003), 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 Gαs but not by forskolin.

PAM is a large protein of 510 kDa that contains a number of potentially functional domains (Guo et al., 1998). Because the N-terminal fragment of PAM, PAM-N (residues 1–1504), contains the region (aa 446-1062; see Fig. 1) that showed could inhibit ACV activity in vitro (Scholich et al., 2001), we investigated whether PAM-N inhibited AC activity in intact cells. As shown in Fig. 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-adrenoceptor agonist isoproterenol. It is notable that 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 in Fig. 2, B and C). In previous in vitro studies, we demonstrated that the full-length PAM and its RCC1-like domain (aa 446-1062) are equipotent in inhibiting AC. However, from the data in Fig. 2, A and B, it would seem that the full-length PAM is less potent than PAM-N at inhibiting AC 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 (data not shown); 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, because the forskolin-stimulated activity was the same whether or not PAM or its derivative PAM-N was expressed (Fig. 2, A and C).

PAM contains two regions, RHD1 (aa 498–740) and RHD2 (aa 874–1065), that are similar to two parts of the RCC1. RCC1 is a guanine nucleotide exchange factor for the small G protein, Ran (Bischoff and Ponstingl, 1991; Carazo-Salas et al., 1999); its structure, a seven-bladed propeller, is similar to the β subunit of heterotrimeric G proteins (Sondek et al., 1996). In previous 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 whether the RCC1-like region of PAM is necessary for inhibition of ACV 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 Fig. 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 submaximal 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. The lack of inhibition of ACV by PAM-NΔRCC1 is not caused by ectopic localization of the protein in some compartment of the cells, which 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 nonspecific manner. The lack of inhibition of ACV by PAM-NΔRCC1 (Fig. 3B) cannot be explained by alterations in the expression of ACV because...
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 to 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 (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 Gas-stimulated endogenous AC activity in Sf9 cell membranes was not inhibited by GST-RHD2 (data not shown). As shown in Fig. 4A, GST-RHD2, but not GST alone, inhibited the ACV activity in Sf9 cell membranes in a concentration-dependent manner. As expected from our previous findings (Figs. 1 and 2) (Scholich et al., 2001), neither GST-RHD2 nor GST alone inhibited the forskolin-stimulated activity of ACV. It is interesting that, compared with the RCC1-like domain (aa 446-1062) (Scholich et al., 2001), the RHD2 domain (aa 861-1075) was less potent at inhibiting ACV activity. These data suggest that additional residues in the N terminus of the RHD2 domain are involved in increasing the potency of the inhibition. We and others have shown that Gas and forskolin together can stimulate the activity of ACV to a greater level than either agent alone (Scholich et al., 1997a). Thus, we investigated whether the RHD2 domain inhibited the activity of ACV that was maximally stimulated with a combination of forskolin and Gas. As shown in Fig. 4B, when Gas and forskolin were present together, the activity of ACV was stimulated to a greater extent than either agent alone. Although the forskolin-stimulated activity of ACV was not inhibited, the GST-RHD2 inhibited the ACV plus forskolin-stimulated ACV activity (Fig. 4B). These data suggest that the GST-RHD2 inhibits Gas-stimulated component of the activity that is maximally stimulated by forskolin plus Gas.
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) (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 Fig. 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 Gs*-stimulated ACV activity was markedly decreased; significant inhibition was observed only at the maximal concentration (300 nM) tested (Fig. 5B). In contrast, the GST-RHD2 inhibited Gs*-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 aa 977 to 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, compared with GST-RHD2, the ability of GST-RHD2CT/22 to inhibit Gs*-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 D. melanogaster (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). It is interesting that human PAM contains two adjacent histidines (His912 and His913) that are located within its RHD2 domain and correspond to His778 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. To begin, we made single and double mutants of GST-RHD2 and examined their ability to inhibit Gs*-stimulated ACV activity. As shown in Fig. 6A, when His912 or His913 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 His912 and His913 with alanine obliterated the ability of RHD2 domain of PAM to inhibit ACV (Fig. 6A). These findings demonstrate that both His912 and His913 play an essential role in inhibition of Gs*-stimulated ACV activity. It is notable that mutation of histidines 912 and 913 to alanine in the GST-RHD2 (GST-RHD2-His912/913A) did not alter its binding to ACV-C2 (Fig. 6B) and did not inhibit ACV activity even at high concentrations (Fig. 6B, bottom). These data confirm the notion that binding of RHD2 to ACV-C2 is not sufficient to inhibit ACV activity. Moreover, the data in Fig. 6 demonstrate that the His912 and His913 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 His912 and His913 residues in the RHD2 region in the context of a larger protein, the experiments shown in Fig. 7 were performed. 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) 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 His912 and His913 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 acids play a pivotal role in modulating Go<sup>s</sup>-stimulated AC activity.

The precise mechanisms by which PAM or its RHD2 domain decreases the ability of Go<sup>s</sup>* to stimulate AC activity are presently unclear. However, because we used the constitutively active mutant of Go<sup>s</sup> (Q213L, Go<sup>s</sup>*) in our assays, it is unlikely that PAM or its RHD2 domain acts as a GTPase-activating protein to decrease the ability of Go<sup>s</sup>* to activate AC activity. Consistent with this notion is our earlier observation that Go<sup>s</sup>*-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 Go<sup>s</sup>* with this region of ACV because the mutation of His912 and His913 in the RHD2 domain that does not alter its binding to C2 is ineffective at altering the ability of Go<sup>s</sup>* 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 Go<sup>s</sup>*-stimulated activity. Because PAM inhibits the isoforms of AC that are inhibited by Go<sup>i</sup> (ACV, ACVI, and ACI), it is tempting to speculate that the protein binds the C2 domain and that, akin to the mechanism of Go<sup>i</sup> inhibition, its N terminus somehow alters the interactions between the C1 and C2 domains to inhibit Go<sup>s</sup>*-stimulated activity. However, this mode of inhibition has to be different from that of Go<sup>i</sup>, because forskolin-stimulated AC activity initiated by Go<sup>s</sup>* is not influenced by PAM-N or full-length PAM.

Fig. 6. Mutation of His912 and His913 to alanine in the RHD2 domain obliterates its ability to inhibit ACV activity but not its binding with the C2 domain of ACV. A, His912 and His913 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 Go<sup>s</sup>*-stimulated ACV activity was monitored as described in the legend to Fig. 5. Data are the mean ± S.E.M., and significance of the differences are shown (n = 6). **, p < 0.01 compared with GST alone; †, not significant compared with GST alone; ††, p < 0.01 between conditions shown by bars. B, the binding of different concentrations (in nanomolar) of GST, GST-RHD2, or its mutant GST-RHD2-H912/913A to 0.15 μM C2 domain of ACV was monitored as described in the legend to Fig. 5 and under Materials and Methods. After 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 Go<sup>s</sup>* (50 nM) stimulated ACV activity in 10 μg of Sf9 membrane was also monitored as described in legend to Fig. 5 and under Materials and Methods. Data presented are the mean ± S.E.M. of three experiments performed in triplicate. *, p < 0.05; **, p < 0.01, Student’s unpaired t test.

Fig. 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). Cells were stimulated with 1 μM isoproterenol (ISO) or 100 μM forskolin (Fsk) for 5 min, and cAMP production was measured as described in the legends to Figs. 1 and 2 and under Materials and Methods. The data presented are the means ± S.E. of either three experiments each performed in triplicate (B) or quadruplicates from a representative experiment (C). **, p < 0.01, Student’s unpaired t test.
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.

Because 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 sphenosine-1-phosphate (Ehnert et al., 2004; Pierre et al., 2004) the other functions of mammalian PAM remain to be elucidated. A clue to the additional functions of mammalian PAM comes from its D. melanogaster and C. elegans homologues RPM-1 and HIW, which have been found to regulate synaptogenesis and neuromuscular junction formation (Schafer et al., 2000; Wang 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 (Scholich et al., 2001), a neuronal AC isoform, suggest that in mammals, PAM may also be important in synaptogenesis. The histidine residues in human PAM (PAM-N) correspond to the histidine in RPM-1 that is necessary to rescue function in the RPM-1 mutant of C. elegans; mutation of these residues obliterates the ability of the protein to inhibit AC, which tempts us to speculate that PAM may modulate synaptogenesis by regulating Gα-stimulated AC activity. The isoforms of AC present in synaptic terminals at neuromuscular junctions remain unknown. 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 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 AC 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 His912 and His913 obliterates the ability of PAM to inhibit AC activity 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 Gα to stimulate AC activity.

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