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GAP Domains Responsible for Ras p21–Dependent Inhibition of Muscarinic Atrial K^+ Channel Currents

GEORGE A. MARTIN, ATSUKO YATANI, ROBIN CLARK, LEAH CONROY, PAUL POLAKIS, ARTHUR M. BROWN, FRANK MCCORMICK

The interaction between the low molecular weight G protein ras p21 and a guanosine triphosphatase activating protein (GAP) uncouples a heterotrimeric G protein (G_k) from muscarinic receptors. Through the use of isolated atrial cell membranes and genetically engineered GAP deletion mutants, the src homology regions (SH2-SH3) at the amino terminus of GAP have been identified as the domains responsible for this effect. Deletion of the domain required to stimulate the guanosine triphosphatase activity of ras p21 relieves the requirement for ras p21 in this system. A model is presented that suggests that ras p21 induces a conformational change in GAP, which allows the SH2-SH3 regions of GAP to function.

THE SIGNALING PATHWAY BETWEEN atrial muscarinic (M2) cholinergic receptors, heterotrimeric G proteins (G_k, probably G_{i2} or G_{i3}), and single muscarinic K^+ channels (K^+[ACh]) can be interrupted in vitro by ras p21 or one of its guanosine triphosphatase (GTPase) activating proteins (GAPs) (*1*). Interruption occurs by uncoupling of the receptor from the G protein rather than uncoupling of the G protein from the channel. The biochemical basis of the effect is not yet understood, but it serves as a sensitive assay for ras p21 and GAP function in a cell-free system. The function of ras p21 depends on interaction with GAP, and vice versa, suggesting that a functional complex forms between these two proteins and that this complex is responsible for interrupting the muscarinic signaling pathway (*1*).

GAP interacts with a number of proteins in vitro. A COOH-terminal domain is responsible for binding H-ras, N-ras, K-ras, and R-ras p21 proteins and for stimulating their GTPase activities (*2*). This domain also binds tightly to the product of the *K-rev1* gene, rap1A p21, but in this case no stimulation of GTPase activity occurs (*3*). GAP binds to phosphoproteins, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors, v-src, and two unidentified phosphoproteins referred to as p190 and p62 (*4–9*). These interactions are mediated by the SH2 regions of GAP (*10, 11*). GAP therefore has the ability to bind two classes of signaling proteins, ras p21 proteins and phosphoproteins, and may serve as a functional link between these signaling pathways. We have now used genetically engineered GAP mutants to identify the GAP domains required for the ras-dependent inhibition of muscarinic receptor coupling to G_k.

The structures of the mutant GAPs used are shown in Fig. 1. GAP17 contains the complete coding sequence of human type I GAP (*12*), the major species of GAP expressed in most tissues (*13*). Three domains are of particular interest: (i) the NH_2 terminus, which contains a hydrophobic region of about 180 amino acids (*12*); (ii) the SH2-SH3 domains, regions of homology with regulatory sequences of non-receptor tyrosine kinases, phospholipase C-γ, the p85 subunit of PI 3′ kinase, and the crk proto-oncogene (*14–19*); and (iii) the catalytic domain, the region sufficient to stimulate ras p21 GTPase activity (*2*). At the COOH-terminus of each GAP mutant, eight amino acids corresponding to the COOH-terminus of SV40 T-antigen were added to facilitate purification of recombinant proteins by the KT3 monoclonal antibody (*20*). GAP16 corresponds to type II GAP, a splicing variant expressed at high concentrations in human placental tissue (*12, 13*). This protein lacks the hydrophobic NH_2-terminus characteristic of type I GAP but is otherwise identical.

The ability of these affinity-purified proteins and of the truncated derivatives (Fig. 1) to inhibit K^+[ACh] currents was tested as described (*1, 21*). The efficiency of full-length type I GAP, which contains the KT3 epitope, was compared with the GAP preparation used in the previous study. The proteins were equally efficient in preventing receptor-mediated channel opening (data not shown). In

G. A. Martin, R. Clark, L. Conroy, P. Polakis, F. McCormick, Department of Molecular Biology, Cetus Corporation, Emeryville, CA 94608.
A. Yatani and A. M. Brown, Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX 77030.

both cases inhibition was due to a reduction in the frequency of opening of the channels; neither mean open time nor unitary conductance was altered. Functioning channels were still present in the patch because application of guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S) produced activation, as described previously (*1*). Hence, the addition of eight amino acids to the COOH-terminus does not affect GAP function in this assay.

GAP240 (Fig. 2) and GAP34 (Fig. 3), which lack the first SH2 site and the SH3 site, were considerably less effective in inhibiting K^+[ACh] currents than GAP17, with GAP240 requiring nearly ten times as much protein as GAP17 to give the full effect (Fig. 2D). GAP14, which consists only of the catalytic region, had little if any effect on K^+[ACh] currents (Fig. 2D) as we have reported (*1*). On the other hand, GAP35, which lacks the second SH2 site, was almost as potent as the wild-type proteins GAP16 and GAP17 (Fig. 3). The effects of type I GAP on K^+[ACh] currents are inhibited by the anti-ras p21 monoclonal antibody Y13-259 (*1*). This observation indicates that GAP requires interaction with ras p21 to exert its effects. We were therefore surprised to find that GAP32 (Fig. 3, B and D) and GAP37, which lack the region of ras p21 interaction, were able to inhibit K^+[ACh] currents. The effects of these proteins, however, could not be blocked by Y13-259 (Fig. 3C).

Figure 3E summarizes the results of this mutational analysis of GAP domains necessary for uncoupling M2 muscarinic receptors from G_k. It appears that the regions of GAP required for this effect are similar to those required for interaction of GAP with phosphoproteins, namely the NH_2-terminal SH2-SH3 region, and that the ability of these regions to exert their effects is mediated by ras p21. On the basis of these results, we propose the following model (Fig. 4). The inhibition of receptor-coupling by GAP requires the first SH2 domain or the SH3 domain. Presumably this coupling involves interaction of these domains with phosphoproteins on the atrial membranes. The COOH-terminal region of GAP (the "ras p21–binding domain") inhibits efficient interaction of GAP with these phosphoproteins. This inhibition is overcome by ras p21, which we propose causes a conformational change that exposes the SH2-SH3 region. A mutant form of GAP that lacks the catalytic domain does not require ras p21 to exert its effects. In this model, the ras-binding domain of GAP is analogous to regulatory domains of protein kinases such as protein kinase C (*22*) and the c-raf protooncogene (*23*), in which removal of the regulatory domain results in constitutive activation of the kinase.

This model leads to several interesting predictions. The first SH2 site of GAP has been shown to bind tightly to activated PDGF and EGF receptors (*10, 11*). We speculate that this interaction, like the interaction of GAP with unidentified components of atrial membranes described here, may be constrained in full-length GAP and that this constraint may also be relieved by ras p21 in the active, GTP-bound state. This scenario would suggest that ras p21 is necessary for efficient binding of GAP to these receptors and possibly to other proteins that interact with the SH2-SH3 region of GAP, such as the GAP-associated proteins p190 and p62 (*8, 10, 11*). We do not know what components of the cell membrane GAP interacts with in the patch-clamp system described here, but phosphoproteins are likely involved, because all known SH2 interactions involve proteins of this class. Consistent with this suggestion is our recent detection of a phosphoprotein of approximately 90 kD in guinea pig atrial cell membrane fractions that binds to the noncatalytic regions of GAP in vitro (data not shown).

In summary, we have determined that the region of GAP that is responsible for uncoupling the muscarinic receptor from G_k in a cell-free system consists of the SH2-SH3 region that directs GAP and other proteins

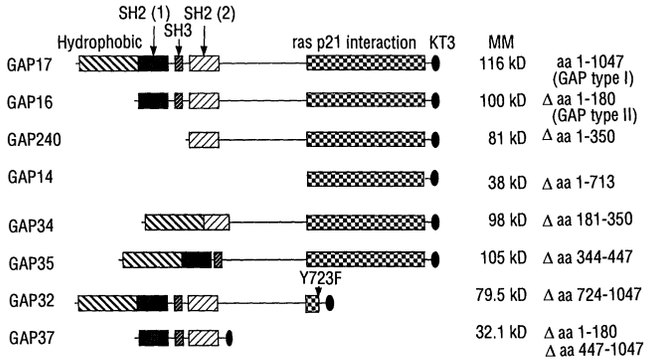

Fig. 1. Representation of the genetically engineered GAPs used in this study. Changes in the amino acid sequence (Δ aa) are noted to the right (MM = molecular mass). GAP14 and GAP37 were constructed by polymerase chain reaction (PCR) with oligonucleotides that had added restriction sites in both the upstream and downstream primers. The downstream primer also contained the sequence encoding the KT3 epitope (*20*). The resulting PCR products were subcloned into the baculovirus vector pAcC4 (*36*). GAP17 and GAP16 were assembled by fusing the cDNA that encodes GAP type I or GAP type II, respectively, to GAP14 at a unique Xba I site. The coding regions of GAP240, GAP34, GAP35, and GAP32 (Y = Tyr and F = Phe) were engineered by oligonucleotide-directed in vitro mutagenesis in M13mp18 (*37*) and then subcloning of the mutated sequences into pAcC4. All alterations in DNA sequences were confirmed by dideoxynucleotide chain-termination sequencing. With the exception of GAP32 and GAP37, all GAPs depicted here can activate the GTPase of ras p21. Recombinant GAPs were immunoaffinity-purified on KT3 affinity matrices (*38, 39*).

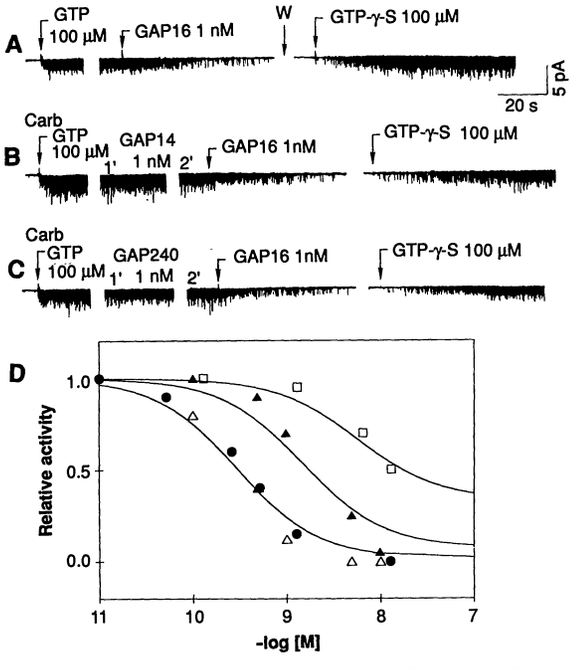

Fig. 2. Uncoupling of M2 muscarinic receptor from atrial muscarinic K^+[ACh] channels by purified, recombinant GAP mutants. Single-channel currents were activated by GTP (100 μM) in the bath (*34*). (**A**) GAP16 was as effective as wild-type GAP or GAP17 but (**B**) GAP14 and (**C**) GAP240 were less effective on block of K^+[ACh] currents. Carbachol (Carb) (10 μM) was added to the patch solution in (B) and (C). Holding potential was −80 mV. Records were direct readouts from the videocassette recorder (VCR) on a chart record. Washing time (W) was 1 min, and the time in minutes (′) after application of GAPs is indicated. (**D**) Concentration-response curves for the effects of (●) GAP17, (△) GAP16, (▲) GAP240, and (□) GAP14 on K^+[ACh] currents ([M] = concentration of mutant). Cumulative currents for 20-s periods were compared before and 60 s after addition of each concentration of GAP. Pooled data were with and without carbachol in the patch pipette and were means from four to six experiments. Percent inhibition at 1 nM for GAP17 and GAP16, GAP240, and GAP14 were 91.88 ± 3.03 (n = 9), 64.0 ± 2.98 (n = 5), and 5.75 ± 2.17 (n = 4).

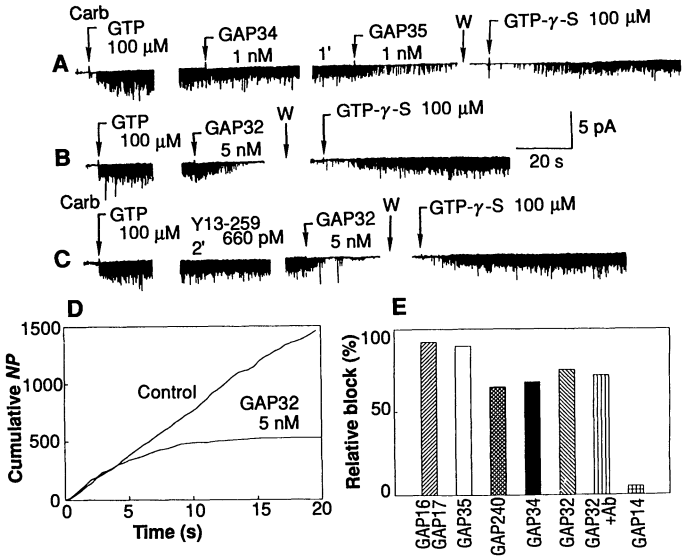

Fig. 3. Relative importance of SH2 domains and COOH-terminus. (**A**) GAP35 was more effective on K^+[ACh] currents than GAP34. (**B**) GAP32 produced a block and (**C**) the block was not prevented by the anti-ras antibody Y13-259. Results with GAP37 were similar to those observed with GAP32, although at twice the concentrations. Recording conditions were identical to those in Fig. 2. (**D**) The change in cumulative *NP* after exposure to GAP32. (**E**) A summary of the block of K^+[ACh] currents produced by mutant GAPs at a concentration of 1 nM. The relative inhibition of K^+[ACh] currents was measured as in (D). GAP32 effects were also compared in the presence of anti-ras (Y13-259) at a concentration of 660 pM (*1*). Data were means from four to eight experiments.

to interact with phosphoproteins. The effects of GAP in this system are dependent on ras p21: we propose that ras p21 causes a conformational change in GAP that relieves a negative constraint that normally prevents GAP from interacting with its target.

The ras-related protein rap1A reverts transformation of mammalian cells by oncogenic K-ras p21 (*24*). The mechanism for this effect may relate to the ability of rap1A to bind tightly to GAP, thus preventing access of ras p21 to this putative effector (*3*). Consistent with this is the recent demonstration that rap1A competitively inhibits the ability of ras p21 and GAP to inhibit muscarinic K^+ channels (*25*). The physiological significance of ras-GAP inhibition is unclear. It is intriguing to imagine that GAP is involved in regulating G-protein function as well as involved in ras p21 and phosphoprotein interactions, but there is, as yet, little biological evidence to support this hypothesis. However, a number of recent reports have shown that G protein signaling pathways activate tyrosine phosphorylation of cellular proteins (*26–28*). We therefore think it likely that physiologically relevant connections between these pathways do exist and that the nature of these connections will soon be elucidated.

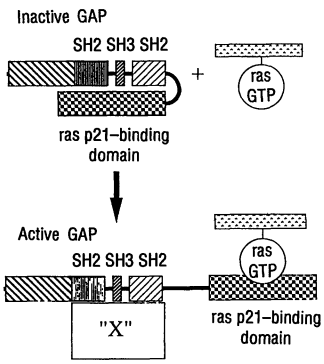

Fig. 4. A model for the inhibition of receptor-coupling by GAP. The first SH2 or SH3 domain is required for GAP action. The COOH-terminal domain of GAP (the "ras p21–binding domain") blocks GAP action. The binding of ras p21 relieves this block, allowing GAP to interact with components of the cell membrane, which results in the uncoupling of the receptor from the G protein.

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21. Single myocytes were dissociated enzymatically from adult guinea pig atria as described (*29*). The pipette and bath solutions were 140 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, and 5 mM Hepes buffer (pH 7.3 with tris base). Carbachol (10 μM) was added to the pipette solution when required. Single-channel currents were measured with the gigaseal patch-clamp method (*30*). The pipette resistance was 5 to 10 megaohms. Single-channel currents were recorded with a List EPC-7 amplifier. Data acquisition and analysis, including calculation of opening probability (*P*) for single-channel currents from *N* channels in a patch (*NP*), have been described (*31, 32*). In brief, currents were stored on videocassette tape for subsequent analysis and were digitized with a 12-bit analog-to-digital converter. To idealize event, the currents were filtered at 1 to 2.5 kHz (−3 dB, four-pole Bessel filter) and sampled at 5 to 10 kHz. Low time resolution records were read from the videotape directly to a strip chart recorder having a −3-dB frequency response of 100 Hz. Nucleotides and proteins were applied to the intracellular face of the excised inside-out patch membrane by the concentration-clamp method. With the concentration-clamp method (*33–35*), K^+ is exchanged after a delay of ~50 ms with a time constant of ~10 ms. To measure concentration-dependent effects of GAP and its mutants, we averaged idealized single-channel currents by measuring the proportion of *P* for *N* channels in the patch pipette, together called *NP*. The quantity *NP* was integrated for variable periods between 200 and 400 ms, and the average value was determined. Accumulated *NP* was determined for a period of 20 s 1 min after the jumps in concentration were made, and the *NP* value was normalized to the *NP* value obtained before exposure to GAP (Fig. 3D). The ratio of average *NP* at each concentration of GAP or GAP mutant to control *NP* was plotted against concentration. The concentration-response data were fit to single occupancy Langmuir absorption isotherms. Carbachol was from Sigma, and GTP and GTP-γ-S were from Boehringer Mannheim (Federal Republic of Germany).
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39. Purification of GAP16 and GAP37 was modified as follows: KT3 antibody (3 mg) was added to the infected-cell lysate, incubated for 2 hours at 4°C, and loaded onto a 1-ml Protein G–Sepharose column over 30 to 60 min at room temperature. The column was washed with 10 to 20 ml of 20 mM tris (pH 8.2), 100 mM NaCl, 0.1% NP-40, and 2 mM β-mercaptoethanol and eluted at room temperature with wash buffer plus KT3 peptide (50 μg/ml) at 4 to 10 column volumes per hour. Fractions containing GAP were pooled and passed through a fresh 0.25-ml Protein G–Sepharose column to remove residual immunoglobulin G.

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