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Muscarinic Receptors Transform NIH 3T3 Cells through a Ras-Dependent Signalling Pathway Inhibited by the Ras-GTPase-Activating Protein SH3 Domain

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Expression of certain subtypes of human muscarinic receptors in NIH 3T3 cells provides an agonist-dependent model of cellular transformation by formation of foci in response to carbachol. Although focus formation correlates with the ability of the muscarinic receptors to activate phospholipase C, the actual mitogenic signal transduction pathway is unknown. Through cotransfection experiments and measurement of the activation state of native and epitope-tagged Ras proteins, the contributions of Ras and Ras GTPase-activating protein (Ras-GAP) to muscarinic receptor-dependent transformation were defined. Transforming muscarinic receptors were able to activate Ras, and such activation was required for transformation because focus formation was inhibited by coexpression of either Ras with a dominant-negative mutation or constructs of Ras-GAP that include the catalytic domain. Coexpression of the N-terminal region of GAP or of its isolated SH3 (Src homology 3) domain, but not its SH2 domain, was also sufficient to suppress muscarinic receptor-dependent focus formation. Point mutations at conserved residues in the Ras-GAP SH3 domain reversed its action, leading to an increase in carbachol-dependent transformation. The inhibitory effect of expression of the Ras-GAP SH3 domain occurs proximal to Ras activation and is selective for the mitogenic pathway activated by carbachol, as cellular transformation by either v-Ras or *trkA*/nerve growth factor is unaffected.

Ras proteins play a pivotal role in both the control of normal cell growth and its malignant subversion (4). They are GTP-binding proteins that function as molecular switches, transmitting signals to downstream effectors when they are in the GTP-bound state (4, 36). The activation state of Ras is determined both by those proteins that catalyze guanine nucleotide exchange (exchange factors) and by those that activate the slow intrinsic GTPase activity of Ras, the GAPs (GTPase-activating proteins) (6, 36, 60, 68). Since GAPs interact with the region of the GTP-bound state of Ras that transmits the signal (2), it has been expected that either the currently defined Ras-GAPs would be effectors for Ras or that any newly described Ras effectors might exhibit GAP activity. Examples of both of these situations have been presented. For example, the Raf protein kinase has recently been shown to interact directly with Ras (24, 44, 61, 63, 65, 74) and to exhibit some GAP activity (65), and the prototypic GAP, p120 Ras-GAP (60), has also been shown to act as a downstream effector for Ras as well as a negative regulator in some systems.

Ras-GAP consists of both a C-terminal catalytic domain and a N-terminal region (N-GAP) that has been shown to uncouple atrial muscarinic receptors (pharmacological subtype M2) from potassium channels (70), modulate cell morphology (40), control gene expression (42), inhibit germinal vesicle breakdown in *Xenopus* oocytes (19), and cooperate with v-Src mutants to cause cellular transformation (15). The inhibition of muscarinic receptor-dependent potassium channel function is particularly interesting, as it has been shown that while neither Ras nor full-length Ras-GAP by itself can exert this action, either the addition of both components together or else addition of just N-GAP can cause uncoupling of the receptors from the channels (37). These data support a model in which the interaction of Ras in the GTP-bound state with the C-terminal catalytic domain of Ras-GAP causes a conformational change in Ras-GAP to expose its N-terminal domains and allow effector function (37).

There are several identified domains within N-GAP, including a pleckstrin homology domain (25, 39) and two SH2 (Src homology 2) domains flanking an SH3 domain (38). SH3 domains have been identified in a large number of signal transduction and cytoskeletal proteins, where they often, but not always, occur in concert with SH2 domains (38). They were originally identified as regions conserved between members of the Src tyrosine kinase family, and the first ideas for their function came from mutations in the SH3 domains of Src which cause oncogenic activation of the protein and thus suggest a negative regulatory role for the domain (48). Subsequently, the Src SH3 domain has been implicated in the binding to and activation of phosphatidylinositol 3-kinase (34, 50, 64). Recently, the three-dimensional structures of SH3 domains from spectrin (45), phosphatidylinositol 3-kinase p85 subunit (28), phospholipase C-γ (27), and Src (72) have been revealed. Despite the relatively low degree of primary sequence identity, a common secondary and tertiary structure of mainly antiparallel β strands assembled into a compact β barrel has been found (27, 28, 45, 72). Further, this structure has been demonstrated to provide a binding pocket for proline-rich peptides (51, 71, 72), motifs which have been shown to

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direct protein-protein interactions with SH3 domains (11, 51). In the examples of the interactions between Src and phosphatidylinositol 3-kinase (50), and a variety of SH3 domains and dynamin (21), such interactions have been associated with activation of catalytic activity. Other putative functions for SH3-dependent protein interactions include subcellular localization (5, 66).

The muscarinic receptor family is composed of five subtypes encoded by distinct genes (m1 to m5) (7, 8, 30, 49). Those subtypes (m1, m3, and m5) that couple through the heterotrimeric G protein Gq to activate phospholipase C (8, 22, 43) are able to morphologically transform NIH 3T3 cells in the presence of the agonist carbachol (22). The relevance of this model system to physiological growth control pathways is supported by the observation that expression of these same muscarinic receptor subtypes in primary cultures of glial cells confers mitogenic responses to carbachol (3). Although carbachol-dependent focus formation has been shown to correlate closely with the activation of phospholipase C, the exact signal transduction pathway mediating transformation is unclear (22, 23, 58). Since Ras and Ras-GAP had previously been implicated in the opening of potassium channels in response to M2 receptors (37, 70), and there is increasing evidence that heterotrimeric G protein-coupled receptors can activate Ras (1, 32, 62, 67), we were interested in exploring the hypothesis that Ras and Ras-GAP participate in muscarinic receptor-dependent cellular transformation.

MATERIALS AND METHODS

Construction of eukaryotic expression vectors. Ras-GAP sequences were amplified from pUC101A (60), kindly provided by F. McCormick, using PCR primers that introduce *Eco*RI sites at both ends of the product DNA or that introduce a *Bam*HI site at the 5′ end and an *Eco*RI site at the 3′ end. Following the restriction site in the 5′ primer was an initiation codon and a purine occupying position −3 (29). A stop codon was introduced in the 3′ primer ahead of the restriction site. Amplified fragments were cloned into the mammalian expression vectors pCMV*neo* and pCH (9), which place inserted sequences under the transcriptional control of the human cytomegalovirus immediate-early enhancer region. The vector pCH is similar to pCMV*neo* except that it adds a nine-amino-acid-residue tag (MYPYDVPDYA, called HA1) with an additional initiation Met residue to the amino terminus of the expressed protein (9). In-frame ligation into the *Bam*HI-*Eco*RI sites adds Gly-Ser just prior to the inserted sequence. Two derivatives of pCH, called pCH3 and pKH3, that encode three reiterated copies of the HA1 tag in series were also constructed. The pKH3 vector additionally contains a simian virus 40 origin of replication. Fusion proteins expressed from these vectors are efficiently immunoprecipitated by the anti-HA1 antibody 12CA5 (Berkeley). A schematic representation of the GAP constructs used in this study is given in Fig. 1.

The vector pCDhm5, which places the coding region of the human m5 receptor gene under the transcriptional control of the simian virus 40 promoter, has previously been described (8). Plasmids encoding various proteins were provided as follows: v-Ras and c-Ha-Ras, D. Lowy; N17-Ras, L. Feig; TrkA, M. Barbacid; p85, L. Cantley; and v-Src, M. Bishop. The sequences encoding the p85 and Src SH3 domains were amplified by PCR and subcloned into pCH as described above.

Mutagenesis of the GAP SH3 domain. The PCR-amplified N-GAP construct (encoding residues 175 to 673) was subcloned into the *Eco*RI site of the phagemid pSELECT-1 (Promega). Oligonucleotide-directed mutagenesis was completed according to the manufacturer's instructions and confirmed by dideoxynucleotide sequencing (Sequenase; U.S. Biochemical) (9).

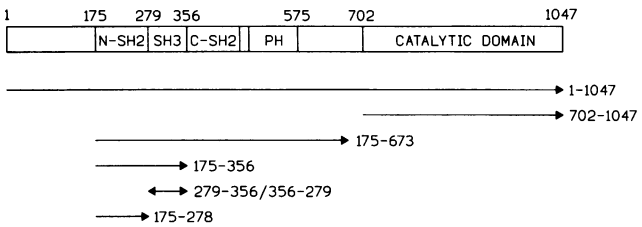

FIG. 1. GAP constructs. Full-length Ras-GAP and the PCR-amplified fragments shown were subcloned into the eukaryotic expression vectors pCMV*neo*, pCH, and pKH3 as described in Materials and Methods. The constructs are identified by the residue numbers of the full-length protein that they encode, and their relationship to the identified domains of Ras-GAP is shown. PH, pleckstrin homology domain.

Cell culture, transfection, and focus formation assays. NIH 3T3 cells were maintained at low passage number and subconfluence in Dulbecco's modified Eagle medium (DMEM) supplemented with 4.5 g of glucose per liter, 4 mM L-glutamine, penicillin-streptomycin, and 10% calf serum (Gibco). For transfection, cells were plated at 2×10^5 cells per 10-cm-diameter plate and grown for 24 h. DNA was added as a coprecipitate with calcium phosphate (54) for a further 24 h. In tests using a β-galactosidase expression plasmid, 20 μg of total DNA per plate was found to give optimal transient transfection efficiency (up to 70%), and so any transfections using less than this amount of plasmid were supplemented to this level with salmon sperm DNA. For coexpression studies, 0.5 μg of pCDhm5, 0.1 μg of pDM69 (*trkA*), or 0.25 μg of pBW1423 (v-*ras*) was mixed with 5 μg of plasmid encoding the specified construct. After transfection, the cells were rinsed and incubated in DMEM supplemented as described above.

To assay for the formation of foci, the medium was changed again on the day after transfection and agonist stimulation was begun. Fresh medium was added every fourth day; after 14 days of agonist treatment, the cells were stained with either cresyl violet or Giemsa stain and the foci were counted. Spontaneous formation of foci was negligible (zero to four foci per plate), although some batches of calf serum cannot be used in this assay since they induce a higher background level (20). The number of foci formed is proportional to the amount of m5 DNA used over a range from at least 10 ng to 1 μg per plate (20).

Immunoblot detection of GAP construct expression. NIH 3T3 cells and COS-7 cells were transfected with 20 and 5 μg, respectively, of the indicated GAP constructs and allowed to approach confluence. The cells were rinsed and scraped into cold phosphate-buffered saline (PBS) and collected by centrifugation. The cell pellet was then boiled in Laemmli sample buffer (31) and passed through a 26-gauge syringe needle to shear the DNA. After separation by polyacrylamide gel electrophoresis and transfer to nitrocellulose, immunoblotting was performed with a polyclonal GAP antiserum (kind gift from F. McCormick), a polyclonal antiserum raised against N-GAP (kindly provided by T. Pawson) (41), or an anti-GAP monoclonal antibody (sc-63, Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated secondary antibodies (donkey anti-rabbit [Amersham] and goat anti-mouse [Jackson ImmunoResearch]) and detection by chemiluminescence (Kirkegaard & Perry).

Clonal survival assays. NIH 3T3 cells were plated at 5×10^5 cells per 10-cm-diameter plate and 24 h later transfected by calcium phosphate precipitation with the indicated plasmids without additional carrier DNA. The antibiotic G418 (Gibco) was added to the medium at 400 μg/ml to select for colonies that had stably integrated the constructs. The cells were selected every 7 days and, at 21 days, were stained with cresyl violet and counted.

Assays of the proportion of GTP-bound Ras. An NIH 3T3 cell line that had previously been selected by morphological transformation in the presence of carbachol following transfection with hm1 (58) was cultured in the presence of G418 (200 μg/ml) but the absence of carbachol, under which conditions it was morphologically normal. Expression of hm1 was confirmed by binding of [^{3}H]*N*-methylscopolamine (NEN) to cell membranes as described previously (22) and shown to be at a level of 5 pmol/mg of protein (approximately 167,000 receptors per cell [22]). Monolayers on 10-cm-diameter plates were grown to confluence, rinsed, and incubated for 24 h in unsupplemented DMEM. To maximize the specific activity of labeling, cells were then rinsed and incubated in phosphate-free DMEM (Gibco) for 30 min prior to the addition of 3 ml of fresh phosphate-free DMEM supplemented with 0.5 mCi of $^{32}P_i$ (NEN) per plate and 20 mM sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4) for 5 h at 37°C. Cells were then stimulated with the agonists shown, lysates were prepared, and the endogenous Ras proteins were rapidly immunoprecipitated with their bound, labeled guanine nucleotides in a procedure adapted from those previously described (9, 18, 55, 73). Briefly, either anti-Ras monoclonal antibody Y13-259, prepared from the hybridoma, or control rat immunoglobulin G (IgG; Jackson) was prebound to washed protein A-Sepharose beads (Sigma) that had previously been treated with rabbit anti-rat IgG (Jackson). Agonist stimulation was terminated by washing three times with ice-cold PBS, and 0.5 ml of cold lysis buffer (50 mM morpholine propanesulfonic acid [MOPS], 0.5% Triton X-100, 150 mM NaCl, 20 mM $MgCl_2$, 100 μM phenylmethylsulfonyl fluoride [pH 7.4]) was added. Lysates were scraped into cold microcentrifuge tubes, insoluble material was removed by centrifugation for 2 min at 4°C, and the supernatants were incubated with antibody-bound beads for 40 min at 4°C with continuous rocking. The immunoprecipitates were washed nine times with 0.75 ml of lysis buffer and then heated at 68°C for 5 min in 25 μl of 20 mM EDTA–1% sodium dodecyl sulfate–1 mM GTP–1 mM GDP. The released, labeled nucleotides were separated by thin-layer chromatography as described previously (9), and the GDP and GTP were quantitated by a Bio-Rad PhosphoImager GS-250. The proportion of Ras in the GTP-bound state was given by the following calculation: % GTP = 100 × cpm in GTP/(cpm in GTP + 1.5 × cpm in GDP) (9).

Analysis of the activation state of the epitope-tagged c-Ha-Ras was similar except that the cells were cotransfected with 10 μg of pCH3, with or without the c-Ha-Ras insert, plus 10 μg of either PCMV*neo*GAP 279-356 or pCMV*neo*GAP 356-279, and then allowed to grow to confluence, the cells were labeled with 1 mCi of $^{32}P_i$ per plate, and the protein-A Sepharose beads were precoupled sequentially to rabbit anti-mouse IgG (Jackson) and anti-HA1 monoclonal antibody 12CA5.

Expression of pCH3c-Ha-Ras. NIH 3T3 cells were transfected with various amounts of plasmid supplemented to 20 μg of total DNA with salmon sperm carrier DNA by calcium phosphate precipitation and grown to confluence. The $HA1_3$-tagged Ras was immunoprecipitated as described above except that after four washes in lysis buffer, the beads were washed twice in lysis buffer with the $MgCl_2$ replaced by 5 mM EDTA and incubated in this buffer plus 10 μCi [α-^{32}P]GTP (NEN) for 30 min at 4°C. $MgCl_2$ was then added to a final concentration of 20 mM, and the immunoprecipitates were washed a further four times in lysis buffer. Further analysis was by scintillation counting and immunoblotting with anti-HA1 monoclonal antibody 12CA5 (9).

RESULTS

Ras function is required for muscarinic receptor-dependent transformation. To investigate whether Ras function is required for muscarinic receptor-dependent transformation of NIH 3T3 cells, two approaches were taken to inhibit Ras. First, the m5 receptor gene in plasmid pCDhm5 was cotransfected into 3T3 cells along with an excess of plasmid encoding full-length GAP (pCMV*neo*GAP 1-1047), plasmid encoding the C-terminal, catalytic domain of GAP (pCMV*neo*GAP 702-1047), or a control plasmid without insert (Fig. 2; Table 1). Overexpression of a catalytically active GAP construct would be expected to inhibit Ras activation by diminishing the ability of the GTP-bound state to be maintained (16, 47). Second, the m5 receptor gene was cotransfected along with a pZip*neo* expression plasmid with or without an insert encoding Ras with a dominant-negative mutation (N17-Ras) (Table 1). Dominant-inhibitory Ras mutants are thought to work by sequestering Ras exchange factors, thus preventing the endogenous Ras from becoming activated (57). Either strategy to inhibit cellular Ras function greatly reduced the ability of carbachol to stimulate foci formation.

Since the dominant-inhibitory N17-Ras mutant can block growth of NIH 3T3 cells (57), it was important to show that carbachol stimulation of cells transfected with both muscarinic receptors and N17-Ras could still induce cell growth, even though transformation was inhibited. The results shown in Fig. 3 demonstrate that the number of G418-selectable colonies arising after transfection with the pZip*neo*N17-Ras construct can indeed be increased by the combination of cotransfection with pCDhm5 and stimulation with carbachol, although neither agent alone has an effect. Further evidence that N17-Ras was acting as expected in our system was provided by testing its effects on focus formation by v-*ras*, which was largely unaffected with only a 17% inhibition, and on focus formation by *trkA*/nerve growth factor (NGF) stimulation, which was inhibited by 73%, i.e., similar to the inhibition of hm5/carbachol-induced transformation (Table 1).

Activation of Ras by muscarinic receptors. Although Ras function is required for hm5-dependent transformation, the possibility remained that this requirement relates to an accessory pathway, perhaps activated by serum, and does not imply that Ras activation is a direct intermediate in the carbachol-induced mitogenic pathway. To address this issue, we assayed the effect of carbachol on the GTP-bound state of Ras in an NIH 3T3 cell line that constitutively expresses a high level of hm1 receptors and exhibits morphological transformation in response to carbachol (58). The results in Fig. 4 demonstrate that carbachol stimulation of these cells is able to significantly activate Ras, inducing an increase in the GTP/GDP ratio similar to that seen in response to the agonist epidermal growth factor (EGF).

Induction of foci by muscarinic receptors can be inhibited by the Ras-GAP SH3 domain. Overexpression of GAP or its C-terminal fragment also inhibits v-*src*-induced transformation (16, 47), but expression of N-GAP is ineffective (16). We were therefore surprised to discover that coexpression of N-GAP (residues 175 to 673) with muscarinic receptors did inhibit carbachol-dependent transformation (Fig. 2; Table 1). Subse-

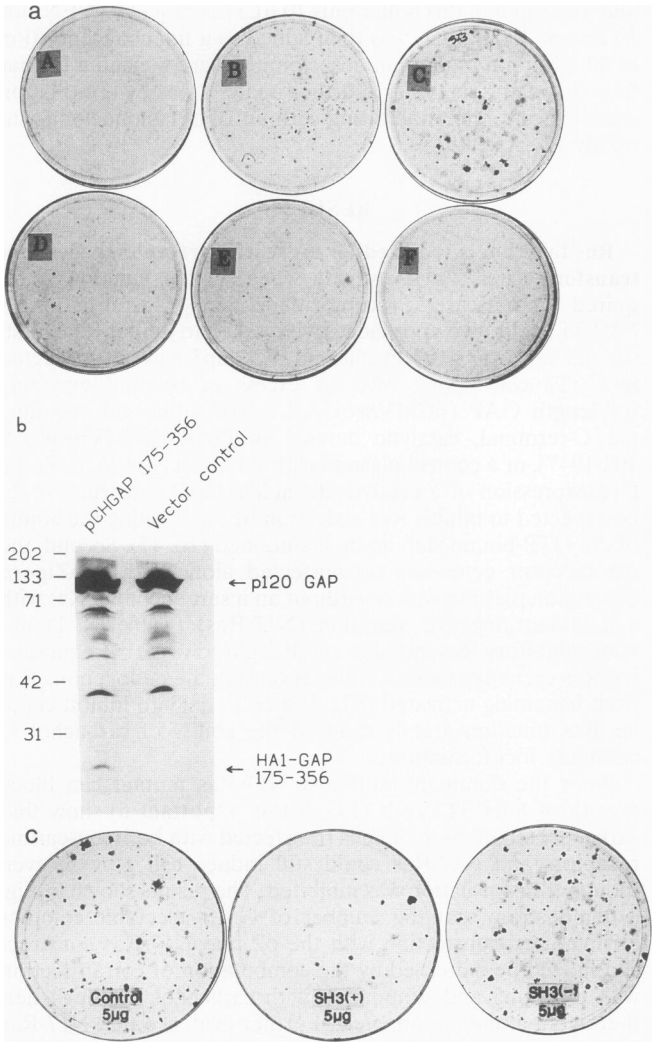

FIG. 2. Suppression of carbachol-induced focus formation by GAP constructs cotransfected with pCDhm5 into NIH 3T3 cells. (a) Focus formation assay with all cultures except A, incubated in 100 μM carbachol for 14 days prior to staining for foci. A to C, transfection with pCDhm5 alone; D, transfection with pCDhm5 plus pCMV*neo*GAP 1-1047 (full length); E, transfection with pCDhm5 plus pCMV*neo*GAP 175-673 (N-GAP); F, transfection with pCDhm5 plus pCMV*neo*GAP 175-356 (N-SH2 and SH3 domains). Quantitation of results from several experiments is given in Table 1. (b) Immunoblot of NIH 3T3 cell proteins, using a polyclonal antiserum against N-GAP (40) following transfection with GAP 175-356 or vector control. Sizes of protein standards in kilodaltons are given at the left. Equivalent results were obtained by immunoblotting with a polyclonal antiserum and a monoclonal antibody against GAP (data not shown). (c) Focus formation assay, 100 μM carbachol treatment of all cultures. Control, transfection with pCDhm5 plus pCMV*neo*; SH3, transfection with pCDhm5 plus pCMV*neo*GAP 279-356; SH3(−), transfection with pCDhm5 plus pCMV*neo*GAP 356-279. Quantitation of results from several experiments is given in Table 1.

quently, inhibition of M1-dependent transformation by N-GAP expression has also been described (69). Since N-GAP includes a number of recognized functional domains, including two SH2 domains flanking an SH3 domain (38) and a pleckstrin homology domain (25, 39), a further series of GAP deletions was engineered into expression vectors (Fig. 1) to test

TABLE 1. Inhibition of hm5 receptor-dependent transformation by cotransfection with N17-Ras or GAP constructs

Construct cotransfected with pCDhm5	Carbachol 100 μM treatment	% Inhibition (mean ± SEM)	*n*
pZip*neo* (vector control)	+	0 (control)	3
pZip*neo*N17-Ras (dominant inhibitory)	+	64 ± 10	3
pCMV*neo* (vector control)	+	0 (control)	5
pCMV*neo*	−	99 ± 1	2
pCMV*neo*GAP 1-1047 (full length)	+	60 ± 17	2
pCMV*neo*GAP 702-1047 (catalytic domain)	+	76 ± 5	2
pCMV*neo*GAP 175-673 (N-GAP)	+	80 ± 13	3
pCMV*neo*GAP 175-356 (SH2 and SH3 domains)	+	65 ± 13	3
pCMV*neo*GAP 279-356 (SH3 domain)	+	67 ± 7	5
pCMV*neo*GAP 356-279 (inverted SH3 domain)	+	8 ± 10	2
pCHGAP 279-356 (tagged SH3 domain)	+	65 ± 11	2
pCHGAP 175-278 (tagged N-SH2 domain)	+	8 ± 1	2
pCHRab3A	+	−18 ± 10	2

in the muscarinic receptor-dependent focus formation assay. It was found that full inhibition was retained in a deletion containing just residues 175 to 356 (comprising the N-SH2 and SH3 domains), expression of which is shown in Fig. 2b. Although it is difficult to draw conclusions about the relative intensities of the induced band and the endogenous p120-GAP (since only a fraction of the culture would be expressing the N-SH2-SH3 construct and the polyclonal antiserum would be expected to recognize the larger protein better), the data are consistent with the GAP 175-356 construct being expressed at a modest level with respect to the endogenous p120-GAP and thus imply a potent effect. In support of this interpretation, the induced N-SH2-SH3 protein, which was detected by three independent anti-GAP reagents, could not be identified by Western blotting (immunoblotting) with monoclonal antibody 12CA5 to the HA1 epitope tag (data not shown), suggesting that the expression level is low, although loss of the epitope tag by proteolysis cannot be excluded.

Division of this region into its separate domains demon-

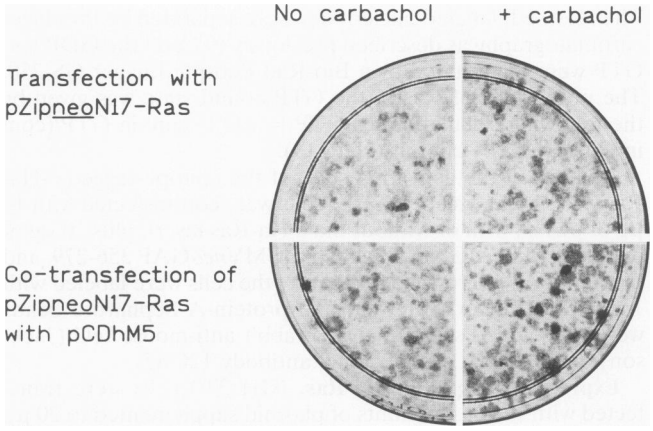

FIG. 3. Carbachol-dependent growth of NIH 3T3 cells cotransfected with N17-Ras and muscarinic receptors. Cells were cotransfected with 10 μg of the indicated plasmids and then cultured for 3 weeks in the presence of 400 μg of G418 per ml with or without 100 μM carbachol as shown.

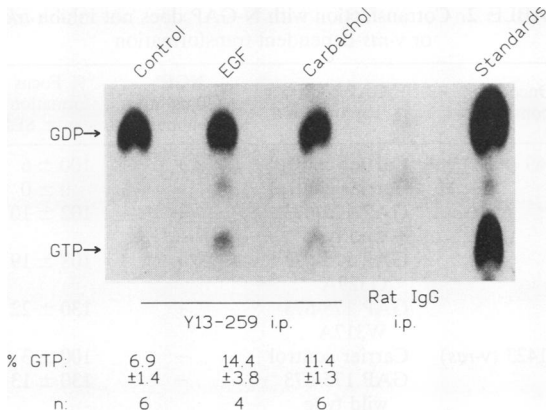

FIG. 4. Activation of Ras by stimulation of muscarinic receptors. Quiescent NIH 3T3 cells that constitutively express hm1 receptors were labeled with $^{32}P_i$ and stimulated with 100 ng of EGF per ml or 100 μM carbachol for 5 min, and immunoprecipitates (i.p.) were prepared by using either anti-Ras monoclonal antibody Y13-259 or control rat IgG. The guanine nucleotides were separated by thin-layer chromatography, and an autoradiograph is shown. Averaged results from a number of such experiments are presented below the autoradiograph as means ± standard errors of the means. Carbachol causes a significant increase in the GTP/GDP ratio on Ras ($P < 0.05$ by two-tailed, paired t test).

strated that the N-SH2 domain (residues 175 to 278) had no effect on carbachol-dependent transformation, while the isolated SH3 domain (residues 279 to 356) was sufficient to suppress focus formation (Table 1). Both the SH3 domain, which was active in the assay, and the SH2 domain, which was inactive, could barely be detected by Western blotting of 3T3 cell extracts (data not shown). This was probably due to a combination of the modest expression levels, the inherent difficulties in electrophoresing, transferring, and detecting fragments of less than 10 kDa, and the division of the epitopes for the polyclonal antisera. Two further negative controls for the focus formation assay were provided by coexpression of the m5 receptor gene along with either Rab3A (a small GTP-binding protein of similar in size to the N-SH2-SH3 construct) or a plasmid that had the isolated SH3 domain inserted in the antisense orientation (pCMV*neo*GAP 356-279) (Table 1; Fig. 2c).

Inhibition of carbachol-dependent transformation as an assay for SH3 domain structure. To exclude the possibility that the suppression of focus formation reflected a nonspecific toxicity of the GAP SH3 domain in this system, NIH 3T3 cells were transfected with either pCMV*neo*GAP 279-356 or pCMV*neo*GAP 356-279 and subjected to selection with the antibiotic G418. Surviving colonies were counted after 3 weeks (Fig. 5). No significant differences in the numbers of colonies were observed, consistent with other results indicating that expression of SH2 and SH3 domains is not toxic to cells (40).

To test whether the suppression of muscarinic receptor-dependent focus formation could be used to identify structural requirements for SH3 domain function, two point mutations were made at highly conserved residues in the SH3 domain of N-GAP. Substitution of alanine for glycine at position 329 is a mutation analogous to the loss-of-function mutation *n2195* in the *Caenorhabditis elegans* protein Sem5 (13), while replacement of trytophan with alanine at position 317 would be expected to perturb the binding pocket for proline-rich peptides that has been deduced from structural studies of several other SH3 domains (27, 28, 45, 71, 72). Expression of these

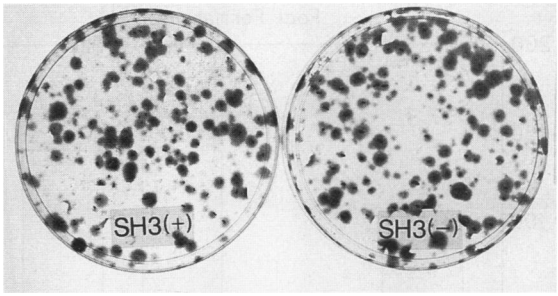

FIG. 5. Lack of cell toxicity of GAP SH3 domain. NIH 3T3 cells were transfected with either 10 μg of pCMV*neo*GAP 279-356 [SH3(+)] or 10 μg of pCMV*neo*GAP 356-379 [SH3(−)] and selected for 3 weeks with the antibiotic G418. Representative plates of duplicate transfections are shown.

constructs in both 3T3 cells and COS-7 cells is shown in Fig. 6. In both cell types, the G329A construct is expressed at a lower level and smaller apparent size than the W317A construct. Although it had been difficult to detect expression of the GAP 175-278 (N-SH2 domain) in 3T3 cells (see above), a triple-tagged N-SH2 domain was easily detectable when expressed in COS-7 cells by using the replication-competent vector pKH3.

Remarkably, expression of the mutant N-GAP constructs not only caused a complete loss of the inhibitory phenotype of the SH3 domain against muscarinic receptor-dependent transformation but actually potentiated transformation, causing a significant increase in the number of carbachol-induced foci (Fig. 7). The mutated GAP fragments were also expressed in the absence of muscarinic receptors to test whether they had any frank transforming potential, but in several experiments, no such activity could be found (data not shown). Interestingly, although these point mutations could abolish the inhibitory activity of the GAP SH3 domain, two other SH3 domains of quite divergent primary sequence from that of the GAP SH3

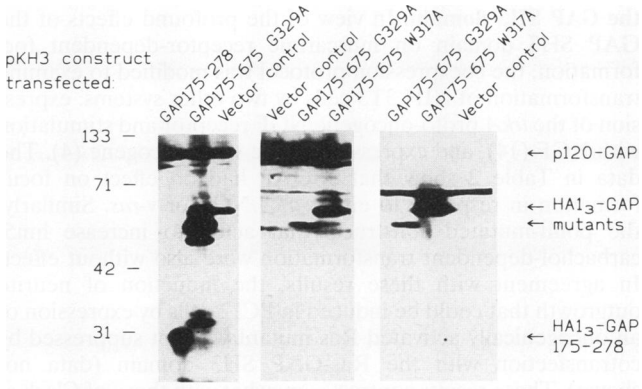

FIG. 6. Expression of GAP constructs in NIH 3T3 and COS-7 cells. Constructs were subcloned into pKH3, which expresses the insert as a fusion protein with a triple HA1 epitope tag under the control of a cytomegalovirus promoter and additionally possesses a simian virus 40 origin of replication. Immunoblots of cell proteins obtained by using either the polyclonal antiserum against N-GAP or anti-HA1 monoclonal antibody 12CA5 are shown. Sizes of protein standards in kilodaltons are given at the left (note that the induced, triple-tagged N-SH2 fragment exhibits a lower electrophoretic mobility than would be predicted).

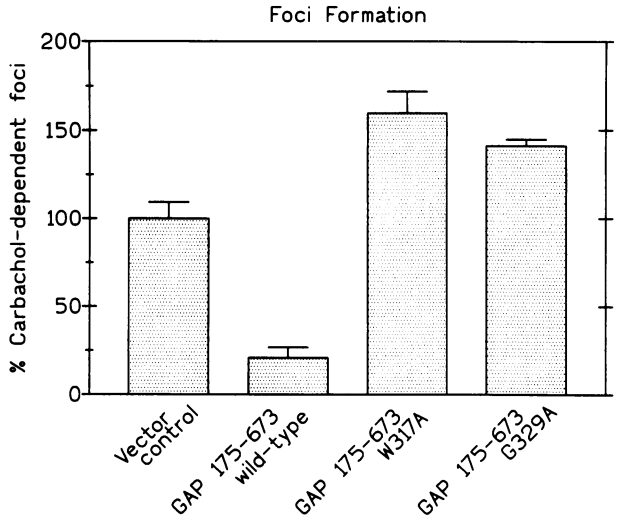

FIG. 7. Point mutations in the Ras-GAP SH3 domain can reverse the suppression of hm5-dependent transformation. NIH 3T3 cells were cotransfected with pCDhm5 and the indicated N-GAP construct in pCMV*neo*. After 2 weeks of 100 μM carbachol treatment, the plates were stained and foci were counted. Data shown are means ± standard errors of the means from four independent experiments conducted in duplicate. The effects of the wild-type N-GAP construct to suppress transformation and of the mutated constructs to enhance transformation were significant ($P < 0.01$ by one-way analysis of variance).

domain, derived from p85 (28) and from Src (72), showed inhibitory activity similar to that exhibited by the GAP SH3 domain (data not shown). These results suggest that the inhibitory target does not show a stringent specificity for a particular SH3 domain, at least when such domains are expressed in isolation. Thus, this assay may be useful for probing structure-function relationships in a variety of SH3 domains.

Selectivity of the suppression of transformation induced by the GAP SH3 domain. In view of the profound effects of the GAP SH3 domain on muscarinic receptor-dependent foci formation, the coexpression protocol was modified to examine transformation of NIH 3T3 cells by two other systems: expression of the *trkA* proto-oncogene/NGF receptor and stimulation with NGF (14), and expression of the v-*ras* oncogene (4). The data in Table 2 show that N-GAP had no effect on focus formation in response to either *trkA*/NGF or v-*ras*. Similarly, the point-mutated constructs that acted to increase hm5/carbachol-dependent transformation were also without effect. In agreement with these results, the induction of neurite outgrowth that could be induced in PC12 cells by expression of an oncogenically activated Ras mutant was not suppressed by cotransfection with the Ras-GAP SH3 domain (data not shown). These results contrast somewhat with those of Clark et al., who find that N-GAP can inhibit v-Ras, but not c-Ras, activity (12).

Inhibition of carbachol-dependent Ras activation by the GAP SH3 domain. To determine whether the SH3 domain-mediated inhibition of transformation occurs upstream or downstream of carbachol-dependent Ras activation, the effect of GAP SH3 domain expression on Ras GTP/GDP ratios was measured. A vector encoding an epitope-tagged c-Ha-Ras protein was constructed, and its expression in NIH-3T3 cells was assayed. Figure 8 shows that by immunoblotting, maximum expression was obtained following a transfection protocol with

TABLE 2. Cotransfection with N-GAP does not inhibit *trkA*- or v-*ras*-dependent transformation

Oncogene construct	GAP construct cotransfected	NGF[a] (30 ng/ml) treatment	% Focus formation (mean ± SEM)	*n*
pDM69 (*trkA*)	Carrier control	+	100 ± 6	3
	Carrier control	−	0 ± 0	2
	GAP 175-673 wild type	+	102 ± 10	3
	GAP 175-673 G329A	+	108 ± 19	3
	GAP 175-673 W317A	+	130 ± 22	3
pBW1423 (v-*ras*)	Carrier control	−	100 ± 3	3
	GAP 175-673 wild type	−	130 ± 13	3
	GAP 175-673 G329A	−	117 ± 18	3
	GAP 175-673 W317A	−	126 ± 22	3

[a] Generous gift from Genentech.

10 μg of the plasmid per 10-cm-diameter plate. In addition to analysis by immunoblotting, the immunoprecipitated, HA1-tagged Ras protein was also tested for its ability to bind [α-^{32}P]GTP. Washed immunoprecipitates representing that obtained from half the material from a 10-cm-diameter plate bound 7,100 cpm (untransfected control), 19,000 cpm (transfection with 5 μg of plasmid), 141,000 cpm (transfection with 10 μg of plasmid), or 37,200 cpm (transfection with 20 μg of plasmid). By both measures, maximal expression of the HA1-tagged c-Ha-Ras was obtained from a transfection protocol using 10 μg of plasmid. Thus, this amount of the *ras* construct was cotransfected with an equal amount of a plasmid either encoding the GAP SH3 domain or with the insert in the reverse orientation into the cells expressing the muscarinic receptors. Under these conditions, more than 70% of the cells that take up one construct would be expected to take up both (26). Since all of the cells are expected to express the muscarinic receptors, carbachol stimulation followed by immunoprecipitation of just the transfected Ras by using the anti-HA1 tag antibody (12CA5) allowed the effect of the GAP SH3 domain on carbachol-stimulated Ras activation to be assessed (Table 3). In cells cotransfected with the control GAP construct of the inverted SH3 domain, carbachol was able to significantly

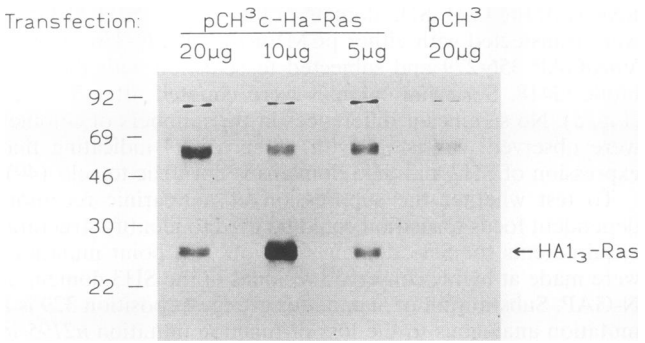

FIG. 8. Expression of HA1-tagged c-Ha-Ras. NIH 3T3 cell proteins were immunoblotted with anti-HA1 monclonal antibody 12CA5 following transfection with the indicated amounts of pCH^{3}c-Ha-Ras or vector control. Sizes of protein standards in kilodaltons are given at the left.

TABLE 3. GTP/GDP ratios on epitope-tagged Ras in 3T3 cells that highly express hm1 receptors

Stimulus	% GTP[a] on immunoprecipitated, tagged Ras[b] (mean ± SEM)	
	pCMV*neo*GAP 356-279 (inverted SH3 domain) cotransfection (n = 4)	pCMV*neo*GAP 279-356 (SH3 domain) cotransfection (n = 5)
Control	6.8 ± 2.7	5.3 ± 1.5
Carbachol, 100 μM, 5 min	17.5 ± 5.5[c]	7.1 ± 3.2
EGF, 100 ng/ml, 5 min	11.0 ± 3.9[c]	15.8 ± 5.0[c]

[a] Radioactivity in 12CA5 immunoprecipitates of labeled control cells transfected with the pCH3 vector was subtracted prior to calculation.

[b] 3T3 cells that constitutively express hm1 were cotransfected with pCH3c-Ha-Ras, encoding $HA1_3$-tagged Ras, and the specified GAP SH3 domain construct.

[c] Increased relative to control, $P < 0.05$, one-tailed t test.

activate the transfected c-Ha-Ras (Table 3) in a manner very similar to its action on endogenous Ras in the untransfected cells (Fig. 4). Cotransfection with the GAP SH3 domain suppressed the ability of carbachol to activate the transfected c-Ha-Ras. Interestingly, EGF was able to activate the c-Ha-Ras whether in the presence of the GAP SH3 domain or the control construct.

DISCUSSION

The results of this study demonstrate that activation of muscarinic receptors expressed in NIH 3T3 cells causes cellular transformation through a pathway that includes, and is dependent upon, the activation of Ras. Such transformation can also be selectively inhibited by expression of the N-terminal, noncatalytic region of Ras-GAP, an effect that resides in the SH3 domain. Expression of the Ras-GAP SH3 domain is sufficient to block both muscarinic receptor-dependent transformation and muscarinic receptor-dependent Ras activation.

This study investigated the effects of both hm5 receptors in focus formation assays and hm1 receptors in the activation of Ras. Although there are structural differences between these receptor subtypes (7, 8, 30, 49), studies on the expressed proteins have found that they couple similarly to second-messenger systems (8) and, with the addition of carbachol, can both transform NIH 3T3 cells (22). The results of this study and the study by Xu et al. (69) show that the abilities of hm5 and hm1 receptors, respectively, to induce focus formation can be inhibited by N-GAP. In stable lines that express approximately 167,000 hm1 receptors per cell, carbachol is able to activate Ras, but in cells expressing fewer than 5,000 hm1 receptors per cell, no such effect of carbachol was found (data not shown). An expression level of at least 20,000 hm1 receptors per cell is required for carbachol to be mitogenic in NIH 3T3 cells (58). We have not been able to isolate stable cell lines that express more than 8,000 hm5 receptors per cell. Thus, the evidence for hm5-dependent Ras activation remains circumstantial, being based on the inhibitory effects of N17-Ras and catalytically active GAP fragments on hm5-dependent focus formation and on the close parallels between the actions of hm1 and hm5 receptors.

It is becoming clear that in addition to their well-established role in signalling from oncogenes or cell surface receptors with intrinsic tyrosine kinase activity, Ras proteins are implicated in signal transduction by those members of the G protein-coupled receptor family that can couple to pertussis toxin-sensitive Gi proteins. Thus, activation of endogenous thrombin (32, 62) or lysophosphatidic acid receptors (62) in astrocytoma cells or Rat-1 and CCL-39 fibroblasts, or activation of ectopically expressed hm2 acetylcholine receptors (67) or α_2-adrenoceptors (1) in Rat-1 fibroblasts, initiates a pertussis toxin-sensitive signal transduction pathway that includes the activation of Ras. Furthermore, stimulation of Gq-coupled hm1 receptors expressed in Rat-1 fibroblasts tends to inhibit Ras activation (53). NIH 3T3 cells, on the other hand, do not display a mitogenic response to thrombin or lysophosphatidic acid (data not shown) but rather exhibit a susceptibility to phospholipase C-induced mitogenesis and transformation. Thus, NIH 3T3 cells can be transformed by microinjection of phospholipase C (56), expression of a constitutively activated Gqα subunit (17), or agonist treatment of cells expressing those muscarinic receptors that couple to Gq/phospholipase C (subtypes m1, m3, and m5) but not those that couple through pertussis toxin-sensitive pathways (subtypes m2 and m4) (22). The identification in this study of Ras activation as a required element in the mitogenic signal transduction pathway initiated by stimulation of hm5 receptors thus adds a new class of heterotrimeric G protein-coupled receptors to those which can potentially signal through Ras activation.

By what route do transforming muscarinic receptors induce Ras activation? One pathway for receptor-induced Ras activation that has recently been defined is that the EGF receptor interacts via phosphotyrosine-SH2 domain association with the coupling protein Grb2, which itself interacts via SH3 domain-mediated association with the exchange factor Sos (6, 10, 35). However, this pathway to Ras activation is not unique. For example, the *trk*/NGF receptor, which also possesses intrinsic tyrosine kinase activity, does not directly associate with Grb2 (59). Further, there are several exchange factors that are distinct from Sos (6). It is possible that the action of *trk*/NGF receptor and cytoplasmic tyrosine kinases such as Src may be mediated through other adaptor proteins, for example Shc (41, 52) or Syp (33). Whether the Ras activation induced by certain heterotrimeric G protein-coupled receptors can be related to this pathway is unknown. In the current study, the activation of c-Ha-Ras tagged with the HA1 epitope can be inhibited by expression of the Ras-GAP SH3 domain. Since the Grb2-Sos interaction is mediated by SH3 domains, it is tempting to speculate that interruption of this interaction may be the route by which the GAP SH3 domain can inhibit both Ras activation and cellular transformation by muscarinic receptors, although such an action might be difficult to reconcile with the lack of effect of the SH3 domain on both EGF-dependent Ras activation and *trkA*/NGF-induced transformation. Thus, it will be interesting to test whether carbachol stimulation of NIH 3T3 cells that constitutively express transforming muscarinic receptors is able to induce translocation of the Grb2-Sos complex to the plasma membrane, as has been shown for EGF in Rat-1 fibroblasts (10).

Previous studies have supported a correlation between the ability of muscarinic receptors to activate phospholipase C and their ability to transform NIH 3T3 cells (22, 58). As our study was being completed, Xu et al. (69) reported that carbachol- and hm1-dependent induction of foci formation in NIH 3T3 cells can be inhibited by coexpression of an N-terminal region of Ras-GAP (residues 1 to 521). The authors show that this inhibition occurs without any effect on the expression or binding properties of the muscarinic receptors and without affecting their ability to activate phospholipase C (69). Our study demonstrates that of the multiple functional domains present in the N-terminal region of Ras-GAP, it is the SH3 domain that is responsible for the inhibition of muscarinic

receptor-dependent transformation and can also prevent the activation of Ras. Taken together, therefore, these results suggest that if phospholipase C activation is indeed required for muscarinic receptor-dependent transformation, which remains to be conclusively demonstrated, then it is likely to either be upstream of Ras activation or else on a separate signal transduction pathway. Although carbachol-dependent phospholipase C activation would be expected to lead to activation of protein kinase C, and NIH 3T3 cells have been shown to exhibit GAP-sensitive phorbol ester responses (46), protein kinase C is unlikely to be an intermediate in muscarinic receptor stimulation of Ras, as carbachol-dependent transformation is independent of conventional protein kinase C isoforms (58).

One difference between the results of Xu et al. (69) and those of the present study is that in their hands, the coexpression of a C-terminal, catalytic domain GAP construct (residues 686 to 1047) did not inhibit transformation by muscarinic receptors (69), a result that the authors suggest may due to a failure of the construct to adequately block Ras function in their system (69). Indeed, since our data show that carbachol-dependent transformation can also be inhibited by the expression of dominant-inhibitory N17-Ras, then overexpression of a catalytically active GAP construct would be expected to inhibit muscarinic receptor-dependent focus formation.

Expression of N-GAP has been reported to induce a number of phenotypic changes in various cell systems, including changes in cell morphology (40), induction of gene expression (42), and cooperation with a myristoylation-defective v-*src* mutant to cause transformation (15). This study and that by Xu et al. (69) extend these observations to include the inhibition of muscarinic receptor-dependent transformation. In addition, this study demonstrates that the entire effect of N-GAP can be reproduced by expression of just the SH3 domain and that the effect occurs upstream of Ras activation. Thus, at least in the present case, it is unclear whether the phenotype gained by cells expressing N-GAP reflects a physiological effector function of the Ras-GAP protein, since, according to the Martin model (37), an effector function inherent in N-GAP should be downstream and thus independent of Ras. Such effector functions of N-GAP have been demonstrated for the uncoupling of atrial (M2) muscarinic receptors from potassium channels (37) and for the inhibition of germinal vesicle breakdown in *Xenopus* oocytes (19). In the latter case, the effector function has also been localized to the SH3 domain (19). Nevertheless, it may be that effector functions of N-GAP are more complicated than a linear model of signalling would allow, with both direct effector functions downstream of Ras and the potential for feedback action in the pathway upstream of Ras. The increased ability of muscarinic receptors to transform NIH 3T3 cells when coexpressed with N-GAP containing a point-mutated SH3 domain could be explained by such a system: the mutations would suppress a feedback inhibition pathway and so induce an increase in signalling. Thus, it will be interesting to determine whether the phenotypes observed following the expression of the N-GAP or its SH3 domain are due to a pharmacological action of the SH3 domain revealed by expression out of context or do indeed reflect a physiological effector function of Ras-GAP.

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