Phosphorylation of Serine 916 of Ras-GRF1 Contributes to the Activation of Exchange Factor Activity by Muscarinic Receptors*  

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The Ras-GRF1 exchange factor is strongly implicated in the control of neuronal Ras. The activity of Ras-GRF1 is regulated by increases in intracellular calcium and the release of Gβγ subunits from heterotrimeric G-proteins. Increases in Ras-GRF1 activity toward Ras that are stimulated by receptors coupled to G-proteins are associated with enhanced phosphorylation of Ras-GRF1 on one or more serine residues. Co-expression of Ras-GRF1 with subtype 1 human muscarinic receptors in COS-7 cells allowed mapping of a carbachol-stimulated phosphorylation site to a region composed of residues 916–976. Site-directed mutagenesis replaced each of the serine residues within this region with alanine and demonstrated that serine 916 is a major site of in vivo phosphorylation of Ras-GRF1 in both COS-7 cells and NIH-3T3 fibroblasts. Serine 916 was a substrate for protein kinase A both in vivo and in vitro, suggesting a novel link between the cAMP and Ras signaling pathways. Carbachol-dependent phosphorylation of serine 916 occurred through a protein kinase A-independent pathway, however. Full-length Ras-GRF1 that contains an alanine 916 mutation was only partially activated by carbachol, suggesting that phosphorylation at residue 916 is necessary for full activation. Phosphorylation of serine 916 in response to forskolin treatment did not, however, increase the activity of Ras-GRF1, indicating that it is not sufficient for activation.

The Ras GTPases are known to play central roles in pathways of cellular growth and differentiation (1). Their function in terminally differentiated cells such as neurons is less clear, but they have been suggested to participate in learning and memory (2). As timed, molecular switches they cycle between active (GTP-bound) and inactive (GDP-bound) conformations under the control of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (3). The balance of the effective GEF and GTPase-activating proteins determines the activation state of Ras proteins. Regulation of the activity or subcellular localization of a specific GEF is now recognized as the major control for Ras activity in many instances (4).

The Ras-GRF1 exchange factor (5), which was also previously known as CDC25Mm (6, 7), is implicated in neurotransmission. Ras-GRF1 is highly expressed in neurons of the central nervous system (8–10) and is present in postsynaptic densities (11). Mice that lack the expression of Ras-GRF1 have defects in the consolidation of memory (12) and in postnatal growth (13). The latter may be due to a decrease in circulating insulin-like growth factor-1 levels that is secondary to changes in the hypothalamus (13).

In contrast to the Sos exchange factors, which couple tyrosine kinase-derived signals to the activation of Ras (14, 15), Ras-GRF1 links heterotrimeric G-proteins (16–19) and calcium signals (20) to Ras. Further, regulation through Sos occurs by translocation of the GEF from the cytosol to its substrate, Ras, at the plasma membrane (21), whereas Ras-GRF1 has not been found to undergo obvious subcellular redistribution (22). Muscarinic acetylcholine receptors, for example, stimulate an increase in the phosphorylation state of Ras-GRF1 that is closely associated with an increase in its exchange activity toward Ras (18). In this study, serine 916 of Ras-GRF1 is shown to be phosphorylated in response to the activation of co-expressed muscarinic receptors. This residue is an in vivo and in vitro substrate for PKA, but another kinase is likely to be responsible for its phosphorylation in response to carbachol stimulation. Phosphorylation of serine 916 is necessary but not sufficient for maximal activation of Ras-GRF1.

EXPERIMENTAL PROCEDURES

Plasmids and Truncation Mutants—Constructs in the pH3 vector (23) that encode full-length Ras-GRF1 and the N-terminal half (residues 1–631, called ΔN) fused with triple hemagglutinin-1 tags at their N termini have been described previously (18). To construct the C-terminal half (residues 632–1262, called ΔN) as a fusion with the epitope-tags, polymerase chain reaction using the primer TCGGATCTGTGGAATTGCTGATTTTATTGACACCTGAGCCAT (which preserves the PstI site) and downstream primer with an EcoRI site, TAAGATTCTACGTGAGAGCCAT, was performed, and the product was subcloned into pH3 and pGEX-2T. All constructs were sequenced. A diagram of the truncation mutants is given in Fig. 1.

Site-directed Mutagenesis—The pKH3 vector was cut with PstI, reacted with T4 DNA polymerase, and then ligated to destroy the PstI site in the multiple cloning cassette (this vector is called ΔPstpKHX). The BamHI/EcoRI fragment encoding Ras-GRF1(900–983) was ligated into ΔPstpKH3 and then cut with PstI and BamHI for insertion of mutated fragments. Site-directed mutagenesis was performed using the two-step megaprimer protocol (24). In the first round, polymerase chain reactions were performed between mutagenic upstream primers (ATGAAAAGCCACTACCAACGCCGAA for serine 907 to alanine, TTTCGAGAGGTTGGTGCCA for serine 916 to alanine, ACACAGGCTTGGCTGAGCCAAT for serine 923 to alanine, CAGCGGATTTGGCTGAGCCAAT for serine 924 to alanine, and TTTCGAGAGGTTGGTGCCA for serine 916 to threonine) and a downstream primer (TGAGCGTTGAGCTGAGCCAAT)
Regulated Phosphorylation of Ras-GRF1 at Serine 916

TATCCTCGATTTACAAAGTT for the region 957–965 of Ras-GRF1. The product of this first reaction (the megaprimer) was then used as the downstream primer in a second polymerase chain reaction with a new upstream primer (GTGCGATAAACCCGACC) for the region 882–887 of Ras-GRF1. The final product was cut with PstI and BstEII for insertion into the vector. All mutated constructs were then sequenced.

To make full-length Ras-GRF1 with the S916A mutation, the BamHI/EcoRI and EcoRI/EcoRI fragments that encode the downstream wild-type region (6) were subcloned into ΔPstPKH3 and then digested with XbaI/SalI. The ΔPstPKH3/Ras-GRF1 plasmid was then digested with PstI (reserving the PstIPstI fragment produced) and then with BstEII. The PstIBstEII fragment including the S916A mutation was then inserted. The resulting plasmid was then again cut with PstI, and the missing PstIPstI fragment was reinserted to restore the full coding sequence. The orientation of the insertion was checked with an EcoRI digest and sequencing of the mutation.

Cell Culture, Transfection, and Metabolic Labeling—Culture and transfection of NIH-3T3/hamster fibroblasts and COS-7 cells using calcium phosphate co-precipitation has been described previously (23). Cultures were labeled with 1 mCi/100-mm dish of [32P]orthophosphate, lysed, and the hemagglutinin-1 Ras-GRF1 was immunoprecipitated as described (19).

Cyanogen Bromide (CNBr) Digestion—Immunoprecipitated, labeled Ras-GRF1 was boiled in Laemmli sample buffer (25), and a small portion was taken for direct SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The majority of the sample was removed into a new tube and precipitated with 10 volumes of acetone at −20 °C for 30 min. After 30 min at maximum speed in a microcentrifuge at 4 °C, the acetone was removed, and the pellet was dried. The sample was then dissolved in 100 μl of 70% (v/v) HCOOH that contained 50 mg/ml CNBr and incubated at room temperature for 90 min. The reaction was dried in a Speed-Vac, dissolved in 100 μl of water, dried again, dissolved in 50 μl of water, and dried again. The sample was dissolved in Tricine gel sample buffer (0.1 M Tris-Cl, pH 6.8, 24% (w/v) glycerol, 8% (w/v) SDS, 0.2% (w/v) Coomassie Blue G-250) and then separated by SDS-PAGE using a Tricine cathode buffer (0.1 M Tricine, 0.1% (w/v) SDS). The gel was then either dried for direct autoradiography or transferred first to a polyvinylidene difluoride membrane to allow subsequent excision of bands and phosphoamino acid analysis (19).

RESULTS

Muscarinic Receptors Stimulate Phosphorylation of Ras-GRF1 at Serine 916—Ras-GRF1 can be activated by carbachol, a muscarinic agonist, in transiently transfected NIH-3T3 fibroblasts that constitutively express hm1, or when co-expressed with hm1 or hm2 in COS-7 cells (18). The increase in exchange activity that can be induced in fibroblasts is closely associated with an increase in the phosphorylation state of Ras-GRF1 (18). In order to map the regulated phosphorylation sites on Ras-GRF1, epitope-tagged Ras-GRF1 was transiently co-expressed with hm1 in COS-7 cells that were then labeled with [32P]orthophosphate and stimulated with carbachol. The immunoprecipitated Ras-GRF1 was then digested with CNBr (Fig. 2A). Carbachol stimulation induced the appearance of a fragment that has a mobility similar to that of the aprotinin moi-

m

A

B

FIG. 1. A schematic view of the Ras-GRF1 constructs used in this study. All of the constructs shown were inserted into the pKH3 vector for expression in mammalian cells as fusion proteins with N-terminal, triple hemagglutinin-1 tags (23). The GRF1(900–983) piece (in both wild-type and alanine 916 forms) was also inserted into pGEX-2T for expression in E. coli as a fusion protein with GST.

FIG. 2. Cyanogen bromide digests of Ras-GRF1 reveal a phosphopeptide that is stimulated by carbachol. A, the Ras-GRF1 constructs shown were co-expressed with hm1 as indicated in COS-7 cells. After labeling with [32P]P, and treatment with 100 μM carbachol for 5 min, the Ras-GRF1 proteins were immunoprecipitated and digested with CNBr. Results shown are autoradiographs of dried gels, with molecular mass markers shown at right. The arrow indicates a fragment that is only seen in the presence of all of hm1, carbachol stimulation, and the C-terminal half of Ras-GRF1. B, phosphoamino acid analysis of the ~6.5-kDa fragment produced in full-length Ras-GRF1 in the presence of both hm1 and carbachol. The position of the standards, indicated at right, was determined by ninhydrin staining. An autoradiograph is shown.

(New England Nuclear) in a final volume of 48 μl. As appropriate, PKI (Calbiochem) was included in the reaction at the indicated concentrations. Reactions were terminated by addition of 4× Laemmli sample buffer and boiling for 5 min prior to separation by SDS-PAGE. To screen the fractions from the Mono-Q column, samples were terminated by binding to nitrocellulose filters (27) with a portion retained for SDS-PAGE to confirm that the radioactivity was incorporated into the assumed substrate. Dried gels were subject to autoradiography and quantification on a phosphorimager.

MonoQ Chromatography—Chromatography was performed at 4 °C with 0.5 mM/min flow rate. The column was first cleaned with 2.5 ml of 4 mM guanidine-HCl in 25 mM Tris, pH 9.0, and then 5 ml of 0.5 mM NaCl in 25 mM Tris, pH 7.4. The column was then equilibrated with 0.5 mM dithiothreitol in 25 mM Tris, pH 7.4, to give a stable baseline (UV at 280 nm) and the sample injected. A linear gradient to 0.5 mM NaCl in equilibration buffer was developed from 3 to 32 min with collection of 1 min fractions.
Regulated Phosphorylation of Ras-GRF1 at Serine 916

A molecular mass marker (6.5 kDa). Inspection of the sequence of Ras-GRF1 predicted that CNBr could produce a 6.5-kDa fragment representing residues 334–386 in the N-terminal half of the protein and a 7.2-kDa fragment representing residues 916–976 in the C-terminal half of the protein. Deletion mutants of Ras-GRF1 that encompass the N-terminal half (residues 1–631, called \( D_N \)) and the C-terminal half (residues 632–1262, called \( D_C \)) showed that it is the C-terminal half that provides this phosphorylated fragment. The 6.5-kDa fragment was excised and subjected to phosphoamino acid analysis, which revealed it to contain phosphoserine (Fig. 2A).

A further deletion mutant was prepared that encompasses residues 900–983 to confirm the conclusion that the region 916–976 of Ras-GRF1 contains a regulated phosphorylation site. In both COS-7 cells, when co-transfected with hm1 (not shown), and in NIH-3T3/hm1 fibroblasts (Fig. 3A), carbachol induced a clear increase in the phosphorylation state of this region of Ras-GRF1. Phosphoamino acid analysis confirmed that phosphoserine was incorporated into this piece (data not shown). Replacement of each of the Serine residues with alanine demonstrated that phosphorylation at serine 916 is necessary for maximal activation of Ras-GRF1 by muscarinic receptors.

Phosphorylation at Serine 916 Is Necessary for Full Activation of Ras-GRF1 by Muscarinic Receptors—To establish whether phosphorylation at serine 916 is required for carbachol-dependent activation of Ras-GRF1, the S916A mutation was inserted into the full-length protein. Exchange factor assays performed on the mutant and wild-type Ras-GRF1 proteins showed that although there was no apparent difference in the basal GEF activity of the two constructs, the S916A mutation prevented full activation by carbachol in NIH-3T3/hm1 fibroblasts (Fig. 4). These results indicate that phosphorylation at serine 916 is necessary for maximal activation of Ras-GRF1 by muscarinic receptors.

Serine 916 of Ras-GRF1 Is a Substrate for PKA—Review of the sequence around serine 916 revealed a consensus site for phosphorylation by PKA (28).

A recombinant protein encompassing residues 900–983 of Ras-GRF1 produced in Escherichia coli as a fusion with GST was therefore used as an in vitro substrate in protein kinase assays. Extracts of NIH-3T3/hm1 fibroblasts that had been stimulated with either carbachol or isobutylmethyl xanthine (IBMX), an inhibitor of cAMP phosphodiesterase and thus indirect activator of PKA (29), were competent at phosphorylation of serine 916 (Fig. 5A). Inclusion of PKI, a selective inhibitor of PKA (30), in the kinase assay was able to completely inhibit the activity present in extracts from IBMX-stimulated cells but only partially inhibit that from carbachol-stimulated cells. This result suggests that carbachol induces a kinase activity toward serine 916 that is distinct from PKA. Note that hm1, although usually characterized as coupling through Gq G-proteins to the activation of phospholipase C (31), have been reported to activate PKA when expressed in fibroblasts (32). Fractionation of the extract from carbachol-stimulated cells by Mono-Q chromatography revealed two peaks of kinase activity toward serine

**Figure 3.** A truncation mutant of Ras-GRF1 is phosphorylated at serine 916. The Ras-GRF1(900–983) constructs were transfected into NIH-3T3/hm1 fibroblasts and then immunoprecipitated after metabolic labeling with \( ^{32}P \) and stimulation with carbachol. The immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose for Western blotting with the 12CA5 monoclonal and enhanced chemiluminescent detection (23), which confirmed equal expression of the constructs (upper panels). The membrane was treated with 1 mM EDTA and 0.05% (w/v) NaN3 in Tris-buffered saline, pH 8.0, to terminate the chemiluminescence, and the autoradiographs were prepared (lower panels).

**Figure 4.** The S916A mutant of Ras-GRF1 is only partially activated by muscarinic receptors. NIH-3T3/hm1 fibroblasts were transfected with constructs that express the full-length Ras-GRF1 proteins shown, serum-starved overnight, and then stimulated with 100 \( \mu M \) carbachol as indicated. Immuno precipitates were prepared and processed for the GEF activity assay with recombinant Ras (18, 19) with data shown as mean ± S.E. of triplicates (A) and a representative immunoblot (sc-224 from Santa-Cruz Biotechnology) to show the recovery of the Ras-GRF1 that was assayed (B).

**Sequence 1**

910EVFRRLMSLANT920

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Regulated Phosphorylation of Ras-GRF1 at Serine 916

DISCUSSION

This study identifies serine 916 of the Ras-GRF1 exchange factor as a target for phosphorylation in vitro in COS-7 cells and NIH-3T3 fibroblasts in response to both the activation of co-expressed muscarinic receptors and the activation of PKA.

916 (Fig. 5B). However, both of these are inhibited by 200 mM PKI (the first peak is reduced by >95%) and the second peak is reduced by >60%) and are thus likely to be two forms of PKA (33). This result suggests that a different purification scheme will be required to identify the carbachol-stimulated kinase.

To confirm the characteristics of the kinase activity in the intact cell, Ras-GRF1(900–983) was transiently expressed in NIH-3T3/hm1 fibroblasts. Treatment of the cells with the cell-permeable and hydrolysis-resistant PKA inhibitor Rp-cAMP-S (34) did not prevent carbachol-stimulated phosphorylation (Fig. 6A). Forskolin, an activator of PKA, in the presence of IBMX was able to strongly stimulate phosphorylation of residue 916 of both the Ras-GRF1(900–983) truncation mutant (not shown) and of full-length Ras-GRF1 (Fig. 6B) as indicated by the appearance of the ~6.5-kDa CNBr fragment. The absence of this fragment from the reactions derived from the S916A mutant confirms the original assignment of this fragment to be due to phosphorylation at residue 916. These results also demonstrate that, just as in the in vitro reactions, serine 916 is apparently a substrate for both PKA and a distinct, carbachol-stimulated kinase in the cell. Note also that there are additional phosphorylation events that are stimulated by carbachol in both the wild-type and S916A forms of Ras-GRF1. For example, there is a clear increase in phosphorylation of a band that has a mobility just below the 21-kDa marker in response to carbachol but not to forskolin.

Phosphorylation at Serine 916 Is Not Sufficient to Activate Ras-GRF1—To test whether phosphorylation at serine 916 was sufficient to activate Ras-GRF1, the stable line of NIH-3T3 cells called 3T3/GRF427, which constitutively expresses Ras-GRF1, was used (19). Treatment of these cells with lysoosphatidic acid stimulates an increase in the activity of Ras-GRF1 (19). Forskolin was unable to induce any detectable increase in the activity of the Ras-GRF1 in these cells (Fig. 7). Similar results were found for Ras-GRF1 that was transiently expressed in NIH-3T3/hm1 fibroblasts (not shown). In addition to activation by Gβγ-dependent phosphorylation, Ras-GRF1 is also able to couple ionomycin-induced increases in cytosolic calcium to increases in mitogen-activated protein kinase activity (20), although ionomycin does not produce an increase in the activity of Ras-GRF1 measured in vitro (20, 22, 35). The combination of ionomycin plus forskolin was therefore tested to establish whether it might serve to stimulate Ras-GRF1, but again, no increase in GEF activity toward Ras was found (Fig. 7).

The effect of in vitro phosphorylation of Ras-GRF1 at serine 916 on exchange activity was also examined. Thus Ras-GRF1 was immunoprecipitated from unstimulated 3T3/GRF427 cells in the absence of phosphatase inhibitors. The Ras-GRF1 was then incubated with baseline and peak fractions from the Mono-Q separation in the presence of ATP and okadaic acid. A GEF assay with recombinant Ras was then performed. No change in the activity of Ras-GRF1 was induced under conditions where serine 916 was phosphorylated in vitro (data not shown).
Regulated Phosphorylation of Ras-GRF1 at Serine 916

Mutation of serine 916 to alanine prevents full activation of the exchange activity of Ras-GRF1 toward Ras. Clearly, however, phosphorylation at serine 916 does not provide a complete explanation of the activation of Ras-GRF1 by G-protein-coupled receptors because it is not, by itself, sufficient to replicate this activation. It is likely that there are additional sites at which phosphorylation occurs in response to agonist stimulation and these events are also required for activation. The absence of these further phosphorylation events would explain the absence of activation in response to forskolin, which may be only able to induce phosphorylation at serine 916. Evidence that there are indeed increases in phosphorylation in response to carbachol at sites that are not so stimulated by forskolin is shown in Fig. 6. Whether phosphorylation at serine 916 in response to a cAMP/PKA signal may contribute to the overall regulation of Ras-GRF1 activity in the cell is unknown, but it is possible that this could provide an additional point of cross-talk between the cAMP and Ras signaling systems (36).

Ras-GRF1 is expressed exclusively in the neurons of the postnatal central nervous system in rodents (10), where, based on the phenotypes of knockout mice that apparently lack expression of Ras-GRF1 (12, 13), it plays a significant physiological role (37, 38). It is reasonable to question, therefore, whether the results from the COS-7 and NIH-3T3 model systems reflect a mechanism that is relevant to physiological events in neuronal signaling. We have previously shown that Ras-GRF1 in neonatal rat brains is a phosphoprotein, the phosphorylation state of which is increased in response to the muscarinic agonist carbachol, and that carbachol increases the exchange activity toward Ras that is present in lysates of rat brains (18). Whether serine 916 of Ras-GRF1 is also a site for phosphorylation in neurons remains, however, to be determined. It should also be noted that Ras-GRF1 may be expressed more widely in human tissues than in rodents (39).

The Ras-GRF2 exchange factor (40) is highly homologous to Ras-GRF1, with much of the difference being short insertion sequences that are present only in Ras-GRF1. These insertions produce the larger size of 140 kDa for Ras-GRF1 rather than 135 kDa for Ras-GRF2. Both Ras-GRF1 and Ras-GRF2 bind calmodulin through their ilimaquinone domains and can couple ionomycin-induced increases in cytosolic calcium into increased activation of mitogen-activated protein kinase (20, 40). It is striking, therefore, that serine 916 of Ras-GRF1 occurs within one of the insert regions and so has no homologous residue in Ras-GRF2. Serine 916 and the adjacent residues are, however, fully conserved between Ras-GRF1 from rodents and humans (5, 6, 41). Because I have found that phosphorylation of serine 916 plays a functional role in the activation of Ras-GRF1 by muscarinic receptors, it will be interesting to test whether Ras-GRF2 is regulated in a similar manner.

Serine 916 lies N-terminal to the CDC25 domain in Ras-GRF1 that has the exchange factor activity for Ras (42) and is just C-terminal to PEST sequences that confer sensitivity, at least in vitro, to proteolysis (43). This region of Ras-GRF2 contains a cyclin destruction box (40). Because phosphorylation may be a signal for protein turnover (44), and because this region of Ras-GRF1 may be concerned with regulation of protein stability, it is possible that phosphorylation of serine 916 also plays a role in the termination of signaling through induced down-regulation of Ras-GRF1.

Another distinction between Ras-GRF1 and Ras-GRF2 had been that I and others (45, 46) had not found any exchange activity toward GTPases of the Rho family in Ras-GRF1, whereas Ras-GRF2 has clear exchange activity for Rac (47). Recently, however, Kiyono et al. (48) have demonstrated that Rac exchange factor activity can be induced in Ras-GRF1 by the co-expression of G-protein βγ subunits. The DBL and pleckstrin homology domains that mediate Rac exchange factor activity (48) are also required for the regulation of the Ras exchange activity of Ras-GRF1 in response to ionomycin (45). This result suggests that control of these two activities may be closely linked. Further, the induction of Rac exchange activity by Gβγ is also associated with an increase in the phosphorylation state of Ras-GRF1, although in this case the event is likely to be phosphotyrosine (48). We previously showed that Gβγ induced an increase in the Ras exchange activity of Ras-GRF1 (18), and this led to the identification of the serine 916 phosphorylation site in this study. In view of the close parallels between the control of the Ras and Rac exchange activities of Ras-GRF1, it is possible that phosphorylation at serine 916 may also participate in the control of Rac exchange activity.

An overall picture of the regulation of Ras-GRF1 is still, therefore, incomplete. Indeed, many GEFs for Ras superfamily GTPases are subject to complex regulation through phosphorylation (49, 50), allosteric interactions (51, 52), and subcellular redistribution (21). GEFs may also play a role in the selection of targets for their substrate GTPases (53). In the case of Ras-GRF1 and its activation by ionomycin, there is now evidence that it can participate in the activation of Raf, the Ras effector, through a mechanism that may be independent of further activation of Ras (46). It is likely that these complexities reflect the underlying function of Ras-GRF1 and other GEFs to serve as key integrators and controllers of signaling pathways with activities beyond the simple control of GTP binding to their substrates.

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