Erk1/2-dependent Phosphorylation of Ga-interacting Protein Stimulates Its GTPase Accelerating Activity and Autophagy in Human Colon Cancer Cells*

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Ga-interacting protein (GAIP) is a regulator of G protein signaling (RGS) that accelerates the rate of GTP hydrolysis by the α-subunit of the trimeric Gα3 protein. Both proteins are part of a signaling pathway that controls lysosomal-autophagic catabolism in human colon cancer HT-29 cells. Here we show that GAIP is phosphorylated by an extracellular signal-regulated (Erk1/2) MAP kinase-dependent pathway sensitive to amino acids, MEK1/2 (PD98059), and protein kinase C (GF109203X) inhibitors. An in vitro phosphorylation assay demonstrates that Erk2-dependent phosphorylation of GAIP stimulates its GTPase-activating protein activity toward the Gαi3 protein (k = 0.187 ± 0.001 s⁻¹, EC₅₀ = 1.12 ± 0.10 μM) when compared with unphosphorylated recombinant GAIP (k = 0.145 ± 0.003 s⁻¹, EC₅₀ = 3.16 ± 0.12 μM) or to GAIP phosphorylated by other Ser/Thr protein kinases (protein kinase C, casein kinase II). This stimulation and the phosphorylation of GAIP by Erk2 were abrogated when serine at position 151 in the RGS domain was substituted by an alanine residue using site-directed mutagenesis. Furthermore, the lysosomal–autophagic pathway was not stimulated in S151A-GAIP mutant-expressing cells when compared with wild-type GAIP-expressing cells. These results demonstrate that the GTPase-activating protein activity of GAIP is stimulated by Erk2 phosphorylation. They also suggested that Erk1/2 and GAIP are engaged in the signaling control of a major catabolic pathway in intestinal derived cells.

Regulators of G protein signaling proteins (RGS) are a family of proteins that control the activity of trimeric G proteins (1–4). More than 20 mammalian RGS proteins have been identified generally by reference to a conserved domain of about 115 amino acid residues known as the RGS box (5). RGS proteins are involved in modulating a variety of cell functions such as proliferation, differentiation, response to neurotransmitters, membrane trafficking, and embryonic development (4, 6). RGS act as negative regulators of several G proteins by accelerating the rate of GTP hydrolysis by the Gα proteins, thereby promoting their association with the βγ dimer (7–9). This GTPase-activating protein (GAP) activity is engaged in the desensitization of signaling by the trimeric G proteins, but it can also speed up the transmission of signals in some cases (10, 11). Recently, the key role of RGS in the regulation of G protein-coupled receptor signaling has been demonstrated in vivo (12, 13). However, recent evidence supports the notion that RGS proteins may be engaged in functions distinct from the regulation of G protein-activity (14).

GAIP (Ga-interacting protein) is an RGS protein, which is known to interact with Gαi3 protein (15). GAIP has been located to the Golgi apparatus membrane and newly budding Golgi vesicles (16, 17) and associated with clathrin-coated vesicles (18), suggesting its potential role in vesicular transport. Recently, it has been demonstrated that posttranslational modifications of RGS can modulate their properties. Palmitoylation of conserved cysteines in RGS boxes has been shown to modify the GAP activity of RGS4 and RGS10 (19). In addition, phosphorylation has been reported to influence the stability and the membrane association of the yeast RGS Sst2 and human GAIP, respectively (20, 21). However, it is not known whether or not phosphorylation could modulate the GAP activity of RGS.

In the present work, we show that the phosphorylation of GAIP is in part dependent upon the activation of the Erk1/2 MAP kinases in the human intestinal HT-29 cells, and both of these events were sensitive to PKC inhibitors and amino acids. Using a panel of Ser/Thr protein kinases in an in vitro assay, we demonstrate that the phosphorylation of GAIP by a recombinant Erk2 stimulated its GAP activity toward the Gαi3 protein when compared with the activity of unphosphorylated GAIP. This stimulation was abolished when serine at position 151 in the RGS domain was replaced by an alanine residue by site-directed mutagenesis.

Previously, we have shown that GAIP and the Gαi3 protein are engaged in a signaling pathway that controls the lysosomal–autophagic route in HT-29 cells (22–24). A hallmark of autophagy in many mammalian cells is its sensitivity to extracellular amino acid levels, which reduce the formation of autophagic vacuoles containing cytoplasmic material destined to lysosomal degradation (25, 26). The inhibition of autophagy by the addition of amino acids was correlated with the inhibition of the Erk1/2 MAP kinases and a low level of GAIP phos-
phorylation in HT-29 cells. By contrast to cells expressing the wild-type GAIP, those expressing the S151A mutant were unable to increase their rate of autophagy in response to amino acid deprivation and Erk1/2 activation.

In conclusion, these results demonstrate that an Erk1/2 MAP kinase-dependent phosphorylation stimulates the GAP activity of GAIP and they also identify GAIP as a target for amino acid-regulated catabolism in intestinal cells.

**EXPERIMENTAL PROCEDURES**

Reverse Transcriptase-PCR and Site-directed Mutagenesis—cDNA were synthesized from mRNA isolated from HT-29 cells by reverse transcription and were used to amplify by PCR full-length cDNA encoding the wild-type GAIP (24). Full-length GAIP mutant S151A was generated by oligonucleotide-directed mutagenesis using the following mismatched oligonucleotide: 5'-GATATCCATCCTGCGCCCAAGGAGGTGCGCTCTGAGCTCCGCGTACAGCTG-3' and reverse primer 5'-CGGGATCCCAAAGGCCTCGGAGGAGGA-3'. Inserts encoding wild-type GAIP and S151A mutant were subcloned into the eucaryote expression vector pcDNA3 (Invitrogen) at the BamHII/XbaI sites. Wild-type and S151A mutant cDNAs were amplified by PCR using the following primers: forward primer, 5'-CGCAAGCTTATGCGGCACCCTGATG-3'; and reverse primer, 5'-CGGGATCCCAAAGGCCTCGGAGGAGGA-3'. PCR products were subcloned into pDNA3.1c/Myc/His-tagged vector (Invitrogen) at the HindIII/BamHI sites. Recombinant proteins were obtained as described previously (24).

Cell Culture and Transfection of HT-29 Cells—HT-29 cells were cultured as described previously (22, 23). His-tagged wild-type GAIP and S151A mutant were introduced into exponentially growing HT-29 cells by the Effectene kit according to the supplier's conditions (Qia-gen). Cells were used 72 h after cell transfection. All experiments were carried out in nutrient-free HBSS medium supplemented with 0.1% BSA, and when appropriate amino acids or drugs were added.

Amino Acid Mixture—The final concentrations of amino acids in the mixture were multiples (4×) of the normal plasma concentrations and were as follows (in μM): asparagine, 60; isoleucine, 100; leucine, 250; lysine, 300; methionine, 40; phenylalanine, 50; proline, 100; threonine, 180; tryptophan, 70; valine, 180; alanine, 400; aspartate, 30; glutamate, 100; glutamine, 350; glycine, 300; cysteine, 60; histidine, 60; serine, 200; tyrosine, 75; ornithine, 100.

Immunoprecipitation and Immunoblotting—Metabolic labeling of HT-29 cells with 0.5 μCi of [32P]orthophosphoric acid (Amersham Pharmacia Biotech) was carried out for 3 h in nutrient-free medium (HBSS), in complete medium, or in the absence or in the presence of amino acids. When appropriate, Erk inhibitors (Calbiochem) were added (H-89, 5 μM; PD98059, 50 μM; PD088959). Cells were collected in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.25 mM sucrose, 5 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 25 mM NaF, 5 mM Na3VO4, 5 μM β-glycerophosphate, 1 mM levamisole, 1 mM para-nitrophenylphosphate, 1.5 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 10 mg/ml pepstatin, 10 mg/ml aprotinin, 1 mg/ml diisopropylfluorophosphatase, 1 mg/ml Dnase I) and after sonication, the lysate was clarified by centrifugation at 50,000 × g for 15 min at 4 °C. Hundred-μg aliquots of proteins were submitted to 9% SDS-PAGE and transferred to nitrocellulose. The membrane was incubated for 1 h in blocking buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% milk. Antibodies phospho-Erk1/2 (1/4000; New England Biolabs) and phospho-p38 MAP kinase (1/2000; New England Biolabs) were incubated overnight at 4 °C in blocking buffer supplemented with 1% nonfat dry milk. After washing in blocking buffer, membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature. Bound antibodies were detected by enhanced chemiluminescence (ECL). The same membranes were then used with anti-Erk1 (1/1000; Santa Cruz) and anti-Erk2 (1/1000; Santa Cruz) or with anti-p38 MAP kinase (1/1000; Santa Cruz) to detect the complete pool of each MAP kinase. The secondary antibody (anti-rabbit) was linked to alkaline phosphatase.

GTase Assays—Single turnover GTase activity measurements were carried out as follows: 250 mM recombinant Go, were loaded with 1 μM [γ-32P]GTP (Amersham Pharmacia Biotech) for 30 min at 30 °C in 50 mM Hepes, pH 7.5, 5 mM EDTA, 1 mM dithiothreitol, 0.1% Lubrol PX, and reactions were next chilled at 4 °C. Free nucleotides were removed by size exclusion chromatography on microspin Sephadex G50. All hydrolysis experiments were done in solution at 4 °C under single turnover conditions. Reactions were started by addition of GAIP containing 15 μM MgSO4, 300 μM unlabeled GTP, and 30 μM to 30 μM recombinant proteins (WT GAIP or S151A mutant) when used. Aliquots (50 μl) were removed at different times, and reactions were stopped by addition of 750 μl 5% Norit activated charcoal in 50 mM NaH2PO4 pH 3.0. Charcoal was removed by centrifugation for 15 min at 12,000 × g, and 400 μl of free phosphate-containing supernatants were counted to determine the amount of P, released per reaction. Zero time point was obtained by adding 30 μl of [γ-32P]GTP in Norit activated charcoal. No GAP activity could be detected by using boiled GAP. The GTase rate constants were calculated by fitting the experimental data to an exponential function: % GTP hydrolyzed = 100(1 e−k(t)), where k is a rate constant for GTP hydrolysis. The results are expressed as the mean ± S.E. of triplicate measurements.
GAIP Phosphorylation and Autophagy

FIG. 1. GAIP is phosphorylated on serine residues. a, upper part. HT-29 cells were radiolabeled with 0.5 mCi of [32P]orthophosphoric acid for 3 h in complete medium, HBSS, or HBSS supplemented with amino acids (see “Experimental Procedures”). GAIP was immunoprecipitated using an anti-GAIP antibody and then submitted to SDS-PAGE. Middle part, Western blot (WB) of immunoprecipitates with an anti-GAIP antibody. Lower part, the ratio [32P]GAIP/WB was determined after scanning. b, phosphoamino acid analysis after acid hydrolysis of immunoprecipitated [32P]GAIP by thin layer electrophoresis. Arrows indicate the mobility of the standards used: PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine. Results are representative of four independent experiments.

RESULTS

GAIP Is Phosphorylated on Serine Residues—Metabolic labeling of HT-29 cells with [32P]orthophosphoric acid followed by immunoprecipitation using an anti-GAIP antibody and SDS-PAGE showed that GAIP was a phosphoprotein (Fig. 1a). Acid hydrolysis of [32P]-labeled GAIP and thin layer electrophoresis revealed only the presence of phosphoserine residues (Fig. 1b). After a 3-h period of nutrient starvation in HBSS, the phosphorylation of GAIP was increased 1.5 times when compared with that observed in cells kept in complete medium. Addition of a mix amino acid to HBSS reduced by 60% the phosphorylation of GAIP, suggesting that an amino acid-dependent signaling pathway is involved in the control of the phosphorylation of GAIP.

Effect of Ser/Thr Protein Kinase Inhibitors on the Phosphorylation of GAIP—Phosphorylation of GAIP was investigated after metabolic labeling with [32P]orthophosphoric acid in the presence of a panel of inhibitors of Ser/Thr protein kinases (Fig. 2). The phosphorylation of GAIP was inhibited by 60% and 70% when bisindolylmaleimide (GF109203X), a broad spectrum PKC inhibitor, and PD098059, an inhibitor of the Erk1/2 pathway, were used, respectively. Inhibitors of casein kinase II (DRB) and p38 MAP kinase (SB203580) have only moderate inhibitory effects on the phosphorylation of GAIP (10% and 25%, respectively), whereas H-89, an inhibitor of PKA, has no effect.

GAIP Phosphorylation Is Controlled by Erk1/2—From the above results, we reasoned that the signaling pathway responsible for the phosphorylation of GAIP is controlled by Erk1/2 and/or PKC. Using antibodies directed against the activated forms of Erk1/2, we showed that amino acids and PD098059 were able to reduce the activation of Erk1/2 (Fig. 3a). By contrast, these products have no effect on the activity of the closely related p38 MAP kinase (Fig. 3b). These results are in agreement with the inhibition of GAIP phosphorylation stimulated by either amino acids or PD098059. It is well known that PKCs can stimulate the Erk1/2 pathway in different cell types (28) including HT-29 cells (29). According to these data, GF109203X greatly impaired the activation of Erk1/2 (Fig. 3a) but has only a moderate effect on p38 MAP kinase (Fig. 3b). This result would explain the inhibitory effect of GF109203X on the phosphorylation of GAIP without totally excluding the possibility that GAIP could be a PKC substrate (see below).

In Vitro Phosphorylation of GAIP by Erk2 Stimulates Its GTPase Accelerating Activity Toward the Gα12 Protein—In order to examine whether the GAP activity of GAIP is influenced by its phosphorylation, we performed an in vitro phosphorylation assay using recombinant GAIP and a panel of Ser/Thr

FIG. 2. Erk1/2 and PKC inhibitors reduce GAIP phosphorylation. Upper part, HT-29 cells were radiolabeled with 0.5 mCi of [32P]orthophosphoric acid for 3 h in HBSS in the absence or in the presence of 100 μM H-89 (PKA inhibitor), 5 μM GF109203X (PKC inhibitor), 100 μM DRB (casein kinase II inhibitor), 10 μM SB203580 (p38 MAP kinase inhibitor), or 50 μM PD098059 (MEK1/2 inhibitor). GAIP was immunoprecipitated using an anti-GAIP antibody and then submitted to SDS-PAGE. Middle part, Western blot (WB) of immunoprecipitates with an anti-GAIP antibody. Lower part, the ratio [32P]GAIP/WB was determined after scanning. (MAP kinase/Erk kinase1/2 inhibitor).
protein kinases (corresponding to the inhibitors used in Fig. 2). We then tested the GAP activity of each recombinant phosphorylated GAIP toward the recombinant Ga$_{a_3}$ protein. Incubation of GAIP with purified kinases in the presence of [γ-32P]ATP revealed labeling by PKC, casein kinase II, and Erk2 but not by PKA and the p38 MAP kinase (Fig. 4a). In parallel control experiments were conducted on histones and myelin basic protein whose substrate characteristics with respect to the different kinases used are well known (see Fig. 4b and “Experimental Procedures”).

The ability of recombinant GAIP incubated with PKC, casein kinase II, or Erk2 to accelerate the GTPase activity of Ga$_{a_3}$ protein was determined during a single round of [γ-32P]GTP hydrolysis experiment. At each concentration of GAIP used (from 30 nM to 30 μM), the rate of GTP hydrolysis (k), corresponding to the P$_i$ liberation as a function of time, was calculated. These values were plotted as a function of GAIP concentration (Fig. 4c). In all assays the Ga$_{a_3}$ protein was present at a concentration of 250 nM.

The intrinsic rate of GTP hydrolysis by Ga$_{a_3}$ was 0.022 ± 0.001 s$^{-1}$. Addition of 30 μM GAIP resulted in acceleration of the GTPase activity by more than 6.5 times (k = 0.145 ± 0.003 s$^{-1}$) with an EC$_{50}$ value of 3.16 ± 0.12 μM. This stimulating effect was abolished when boiled GAIP was used. The casein kinase II- and PCK-mediated phosphorylation of GAIP did not change the rate of GTP hydrolysis when compared with that of the recombinant GAIP (k = 0.146 ± 0.002 s$^{-1}$, EC$_{50}$ = 3.15 ± 0.11 μM and k = 0.158 ± 0.002 s$^{-1}$, EC$_{50}$ = 3.09 ± 0.12 μM, respectively). However, an increase in the rate of GTP hydrolysis and a 3-fold reduction of EC$_{50}$ for GAIP were observed when phosphorylation was effectuated by Erk2 (k = 0.187 ± 0.01 s$^{-1}$, EC$_{50}$ = 1.12 ± 0.1 μM) as compared with the unphosphorylated recombinant GAIP. The calculation of the estimated stoichiometry of in vitro Erk2 phosphorylation of GAIP indicates an average incorporation of 0.4 mol of phosphate/mol of protein, suggesting the presence of a single Erk1/2 phosphorylation site.

**Mutation of Ser$^{151}$ Abolishes Both the Erk2 Phosphorylation of GAIP and Its Stimulation of the GAP Activity**—The primary sequence of GAIP contains two consensus sites for PKC and seven consensus sites for casein kinase II (15). However, several SP motifs that are potential phosphorylation sites for MAP kinases are also present. Among them, serine 151 in the RGS domain appears to be the most appropriate consensus site for Erk1/2 (ILSP) (30). This sequence is highly conserved among the GAIP subfamily (GAIP, Ret-RGS1, and RGSZ1) and absent among the five other RGS subfamilies (5) (Fig. 5a). For this reason we performed site-directed mutagenesis on Ser$^{151}$ in order to generate a recombinant S151A GAIP mutant. Both GAIP and the S151A GAIP mutant were then examined in an in vitro phosphorylation assay.

The absence of phosphorylation of S151A GAIP mutant by Erk2, shown in Fig. 5b, is not a consequence of gross modifications of the protein structure because: (i) the GAIP activity of the S151A GAIP protein is close to that of the wild-type GAIP (k = 0.119 ± 0.002 s$^{-1}$, EC$_{50}$ = 4.17 ± 0.09 μM versus k = 0.145 ± 0.003 s$^{-1}$, EC$_{50}$ = 3.16 ± 0.12 μM), (ii) PKC phosphorylates both the S151A GAIP mutant and wild-type GAIP in a similar manner (Fig. 5b), and (iii) the heat-denatured S151A GAIP mutant is not a substrate for Erk2 (data not shown).

After incubation with PKC or Erk2, the rate of GTP hydrolysis observed in the presence of S151A GAIP mutant was reduced compared with that observed with phosphorylated wild-type GAIP (Fig. 5c).

These data strongly suggest that phosphorylation of serine 151 residue by Erk2 is required for the increase of the GAP activity of GAIP.

**Erk1/2 and GAIP Are Engaged in a Signaling Pathway That Controls Autophagy in HT-29 Cells**—We have reported that the autophagic pathway is dependent upon the activity of the Ga$_{a_3}$
FIG. 5. Effect of the mutation S151A on the phosphorylation of GAIP by recombinant Erk2 and its GAP accelerating activity. a, schematic representation of GAIP containing the conserved sequence including the phosphorylation site of Erk2. b, upper part, 5 μg of recombinant GAIP (WT) or S151A mutant (S151A) were phosphorylated with 1 μCi of [γ-32P]ATP in the presence of recombinant PKC or Erk2 as described under “Experimental Procedures”; lower part, Western blot (WB) of recombinant GAIP using an anti-GAIP antibody. c, GAP activity of Goα3 (250 nm) was measured in the presence of increasing concentrations (30 nm to 30 μM) of recombinant GAIP (WT, inset), S151A mutant (S151A, inset) or phosphorylated forms of each of the recombinants obtained after in vitro phosphorylation using Erk2 (WT/Erk2 and S151A/Erk2) and PKC (WT/PKC and S151A/PKC). GAP accelerating activity and k determinations were measured as described in Fig. 4.

Discussion

Several studies have shown that the activity of RGS is controlled at the transcriptional level (4). Posttranslational modifications of RGS (including palmitoylation and phosphorylation) have been reported to be involved in their cellular localization and stability (19–21, 31). To our knowledge, our results provide the first evidence that in vitro phosphorylation of GAIP by the Erk2 MAP kinase increases its GAP activity toward the Goα3 protein. Substitution of Ser151 by Ala abrogates this stimulation. The GAP activity of S151A GAIP is comparable to that observed with unphosphorylated wild-type GAIP. This result strongly suggests that this GAIP mutant is still functional and able to interact with the Goα3 protein. Ser151 is located in a loop connecting helices V and VI of GAIP in its RGS domain (32). Crystallographic data have shown that this loop is involved in the interaction of RGS4 and the Goαi protein (33). A critical residue in this loop is RGS4-Asn128. A serine residue (Ser156) in GAIP occupies this position. This characteristic defines a subfamily of RGS proteins, which includes GAIP, RET1-RGS, and RGSZ1 (5). The sequence upstream of Ser156 is also conserved in this subfamily Ile-Leu-Leu-Leu-Pro157, whereas in other RGS subfamilies this tetrapeptide is not conserved. The determination of the soluble structure of GAIP by NMR techniques has suggested that, upon binding to Goαi, conformational rearrangements of the loop V-VI may facilitate the formation of electrostatic interactions that stabilize protein and GAIP in HT-29 cells. To study the relationship between GAIP phosphorylation and macroautophagy, we have measured the autophagic sequestration of the cytosolic enzyme LDH in sedimentable material (Table I) and the degradation of long-lived [14C]valine-labeled proteins (Fig. 6) in HT-29 cells transfected with His-tagged expression vectors containing either the wild-type GAIP cDNA or the S151A GAIP mutant cDNA. According to our previous studies (24), the overexpression of wild-type GAIP stimulates both autophagic parameters shown in Figs. 1 and 2 on the effect of amino acids and PD098059 on phosphorylation of GAIP and Erk1/2 activation.

By contrast to the stimulatory effect of wild-type GAIP on autophagy, S151A GAIP failed to increase the rate of autophagy in the absence of amino acids under conditions where Erk1/2 MAP kinases were activated in S151 GAIP-expressing cells (data not shown) and the same level of transfected proteins were expressed (Table I).

Table I

| His-tagged vector | GAIP expression | Autoautophagic sequestration of LDH (%) | HBSS | HBSS + amino acids | HBSS + PD098059 |
|-------------------|----------------|---------------------------------------|------|-------------------|-----------------|
| Empty             | 1.45 ± 0.97    | 2.15 ± 0.87                           | 2.31 ± 0.58 |
| WT GAIP           | 9.52 ± 1.55    | 2.75 ± 0.51                           | 2.94 ± 0.58 |
| S151A GAIP       | 4.25 ± 0.51    | 2.07 ± 0.35                           | 2.31 ± 0.40 |

The ratio of overexpressed GAIP (WT or S151A mutant)/endogenous GAIP was calculated after scanning of a Western blot using an antibody directed against GAIP. The overexpression of His-tagged WT GAIP or His-tagged S151A GAIP was then detected by Western blot using an antibody directed against His tag.

The values reported are the mean ± S.D. of four determinations.

FIG. 6. Macroautophagy is regulated by Erk1/2 activity. The rate of [14C]valine-labeled long-lived protein degradation was measured in parental and transfected cells incubated in HBSS. Transfections were done using pCIn 3.1-C-Myc-His-tagged vector containing either the wild-type GAIP (WT) or S151A mutant (S151A), or the empty vector. Experiments were performed in the presence or in the absence of amino acids or 50 μM PD098059. Data are expressed as the percentage of cellular protein degraded after 4 h. Results are representative of four independent experiments.

Ser-Pro157, whereas in other RGS subfamilies this tetrapeptide is not conserved. The determination of the soluble structure of GAIP by NMR techniques has suggested that, upon binding to Goαi, conformational rearrangements of the loop V-VI may facilitate the formation of electrostatic interactions that stabilize protein and GAIP in HT-29 cells. To study the relationship between GAIP phosphorylation and macroautophagy, we have measured the autophagic sequestration of the cytosolic enzyme LDH in sedimentable material (Table I) and the degradation of long-lived [14C]valine-labeled proteins (Fig. 6) in HT-29 cells transfected with His-tagged expression vectors containing either the wild-type GAIP cDNA or the S151A GAIP mutant cDNA. According to our previous studies (24), the overexpression of wild-type GAIP stimulates both autophagic parameters shown in Figs. 1 and 2 on the effect of amino acids and PD098059 on phosphorylation of GAIP and Erk1/2 activation.

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the RGS protein structure (32). Whether phosphorylation of Ser^{151} could directly interfere with the residues of Go, actively involved in GTP hydrolysis or stabilize GAIP structure to optimize its GAP activity remains to be elucidated by structural studies.

According to the presence of potential phosphorylation sites in its sequence, GAIP was in vitro phosphorylated by casein kinase II and PKC but not by PKA and p38 MAP kinase. These results are in good agreement with the inhibition profile of GAIP phosphorylation observed in vivo. Recently it has been reported that the casein kinase II-dependent phosphorylation of GAIP on Ser^{24} (outside of RGS domain) could regulate its membrane association (21). Although Ser^{151} is a potential phosphorylation site for PKC, it is not likely to be a substrate for this kinase in vitro because the PKC-dependent phosphorylation of the S151A GAIP mutant was similar to that of the wild-type GAIP. However, it is interesting to note that a PKC site is located in the C-terminal part of GAIP (Thr^{201}) in the vicinity of a GIPC, a PDZ domain-containing protein, interacting site (34). These data suggest that depending on the acting Ser/Thr protein kinase, the occupancy of different phosphorylation sites regulates different functional properties of GAIP.

Following up the above reported results, we have shown that GAIP is phosphorylated in an Erk1/2-dependent manner in a cellular environment. This adds a novel mammalian non-nuclear substrate for Erk1/2 MAP kinases (35). The list of non-nuclear MAP kinase substrates includes several proteins involved in the interruption of G protein signaling pathways (members of G protein-coupled receptor kinases and arrestins (20, 36). This suggests that MAP kinases can act as feedback regulators of trimeric G protein signaling. The recent demonstration of this feedback control of MAP kinases on G protein signaling via RGS in yeast emphasizes the importance of this regulation loop, which has been conserved during evolution (20).

Finally, our work concerns the signal control of the macroautophagic pathway. Previously, we have demonstrated that GAIP is a regulator of the G_{13} protein-dependent macroautophagic pathway in intestinal derived HT-29 cells (24). A hallmark of macroautophagy in many mammalian cells including HT-29 cells is to be inhibited by amino acids (26). This inhibition has been demonstrated to be dependent upon the phosphorylation of the ribosomal S6 protein by the activation p70S6 kinase in rat hepatocytes (37). This signaling pathway is also operative in HT-29 cells. Here we report that amino acids can control autophagy by inhibiting Erk1/2-dependent GAIP phosphorylation. This control is dependent upon the presence of Ser^{151} in GAIP suggesting that the Erk1/2-dependent phosphorylation of Ser^{151} accelerates the GTP hydrolysis by the G_{13} protein. This would be in line with our previous data showing that the GDP-bound form of the G_{13} protein increases the rate of autophagy (23). In HT-29 cells amino acids have a coordinated inhibitory effect on autophagy by activating the p70S6 kinase and inhibiting the Erk1/2 pathway. The mechanism by which amino acids control the Erk1/2 pathway in this cell line remains to be investigated. The control of macroautophagy by the p38 MAP kinase has been reported in rat hepatocytes in response to change in cell volume (38). Although care must be taken in extrapolating data from different experimental models, a role for the MAP kinase family in the control of the signaling of a major catabolic pathway could be a new function for these kinases.

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