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The inhibition of the GTPase activating protein - Ha-*ras* interaction by acidic lipids is due to physical association of the C-terminal domain of the GTPase activating protein with micellar structures

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The effects of fatty acids and phospholipids on the interaction of the full-length GTPase activating protein (GAP) as well as its isolated C-terminal domain and the Ha-*ras* proto-oncogene product p21 were studied by various methods, viz. GTPase activity measurements, fluorescence titrations and gel permeation chromatography. It is shown that all fatty acids and acidic phospholipids tested, provided the critical micellar concentration and the critical micellar temperature are reached, inhibit the GAP stimulated p21 GTPase activity. This is interpreted to mean that it is not the molecular structure of acidic lipid molecules *per se* but rather their physical state of aggregation which is responsible for the inhibitory effect of lipids on the GTPase activity. The relative inhibitory potency of various lipids was measured under defined conditions with mixed Triton X-100 micelles to follow the order: unsaturated fatty acids > saturated acids ≈ phosphatidic acids ≥ phosphatidylinositol phosphates >> phosphatidylinositol and phosphatidylserine. GTPase experiments with varying concentrations of p21 and constant concentrations of GAP and lipids indicate that the binding of GAP by the lipid micelles is responsible for the inhibition, a finding which was confirmed by fluorescence titrations and gel filtrations which show that the C-terminal domain of GAP is bound by lipid micelles.

Key words: GAP/GTPase/lipid micelles/p21/*ras*

Introduction

The Ha-*ras* proto-oncogene product p21 participates in the transduction of proliferative signals by a mechanism that is largely unknown. It is generally accepted that p21 is active when GTP is bound and becomes inactive upon hydrolysis of GTP to GDP, a reaction which is stimulated by the GTPase activating protein (GAP) (Trahey and McCormick, 1987), which itself may be the target of p21 (Cales *et al.*, 1988; Adari *et al.*, 1988) or may regulate the interaction of p21 with its target (for reviews see Hall, 1990; McCormick, 1990). Exchange factors which stimulate the p21-GDP dissociation have been identified (Downward *et al.*, 1990b; West *et al.*, 1990; Wolfman and Macara, 1990; Huang *et al.*, 1990). It has been speculated that these exchange factors in analogy to the hormone dependent adenylyl cyclase activation, may be the upstream targets of mitogenic signals which are transmitted via the ras protein to a still elusive downstream effector. On the other hand it has been shown that p21-GTP accumulates intracellularly in response to stimulation with growth factors (Downward *et al.*, 1990a; Satoh *et al.*, 1990a,b) and, furthermore, that growth factor receptors interact with and phosphorylate GAP (Molloy *et al.*, 1989; Kazlauskas *et al.*, 1990; Ellis *et al.*, 1990; Kaplan *et al.*, 1990; Margolis *et al.*, 1990) and thereby may modulate its activity towards p21 resulting in stimulation of the formation of the active GTP-bound form of p21. This sequence of interactions might couple a mitogenic signal —binding of a growth factor to its receptor—to an activation of p21. In addition, mitogenically responsive lipids have been demonstrated to inhibit the GAP stimulated p21 GTPase (Tsai *et al.*, 1989a,b) and it was speculated that this inhibition might be important in the regulation of the p21 activity during mitogenic stimulation.

In a project dealing with a search for low mol. wt compounds which interfere with the action of p21 and/or its interaction with other proteins we had screened a *Streptomyces* culture collection and found some strains to contain compounds to interfere with the p21-GAP interaction. These were identified to be oleic and linoleic acid. To find out whether the inhibitory effect observed is due to binding of these and related compounds to GAP or p21 we have carried out a biochemical and biophysical analysis of the ternary system comprising GAP, p21 and various lipids. The results of this analysis suggest that the inhibitory effect is common to unsaturated fatty acids and acidic phospholipids when present in micellar structures and is due to binding to and sequestration of GAP.

Results

The analysis of the effects of various lipids on the p21-GAP interaction was carried out with homogeneous preparations of p21 overexpressed in and isolated from *Escherichia coli*, human GAP overexpressed in and isolated from a baculovirus infected cell culture, as well as a truncated version of human GAP (amino acids 714-1047) overexpressed in and isolated from *E.coli*. Some experiments were carried out with a crude preparation of porcine GAP. Two approaches were chosen to analyze the effects of lipids on the p21-GAP interaction. In the first approach the GAP stimulation of the GTPase activity of p21 was determined in the presence of various lipids. The other approach consisted of the identification of a physical interaction between various lipids on the one hand and GAP or p21 on the other.

Inhibition of GAP stimulated p21 GTPase by unsaturated fatty acids in pure micelles

The GAP stimulated p21 GTPase was measured in the presence of increasing amounts of various fatty acids by a modified nitrocellulose filter assay (Gibbs *et al.*, 1988).

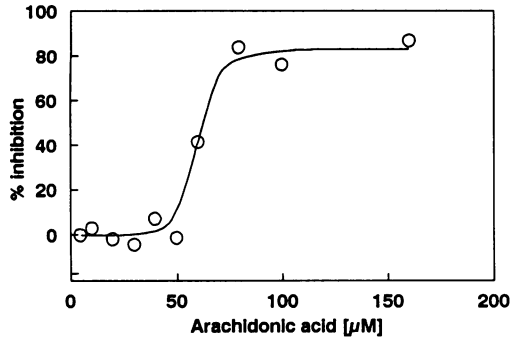

Fig. 1. Dependence of the GAP stimulated p21 GTPase on inhibitor concentration. 0.2 μM p21−[γ-^{32}P]GTP and ~5 nM GAP from porcine brain (the concentration is a rough estimate based on a comparison of the activity of the crude porcine GAP preparation with the homogeneous human GAP preparation) and increasing concentrations of arachidonic acid were incubated in 20 mM HEPES, pH 7.6, at 25°C. The amount of p21−GTP remaining after 30 min was determined by the nitrocellulose filter assay. 0% and 100% inhibition is defined as the value obtained in the presence or absence respectively, of GAP without added fatty acid.

Figure 1 shows that arachidonic acid (C20:4) at concentrations <50 μM does not significantly affect the GAP stimulated p21 GTPase activity, but at concentrations >100 μm suppresses the GTPase activity to levels observed in the absence of GAP. Similar steep transitions from no to maximum inhibition of stimulation were observed for palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2) and docosahexaenoic acid (C22:6). A more shallow transition was measured for linoleic acid (C18:3). With myristoleic acid (C14:1) and elaidic acid (C18:1 *trans*), as well as all saturated fatty acids tested, namely palmitic acid (C16:0), stearic acid (C18:0) and arachidic acid (C20:0), no effect on the stimulation of the GTPase reaction was observed up to the highest concentrations tested (200 μM).

The sigmoidal shape of the concentration dependence suggests that the inhibition of the p21−GAP interaction by some fatty acids follows a cooperative mechanism. A likely explanation for this result is that the inhibitor is not the fatty acid molecule but an aggregate of fatty acids. As shown in Table I the critical micellar concentration (CMC: here operationally defined as the concentration at which aggregation of lipids occurs, for details see Tanford, 1980), as measured under the conditions of our assay (Figure 2), closely matches the concentration of half maximal inhibition (IC_{50}). Those fatty acids which do not form micelles at concentrations <200 μM or at temperatures <37°C, like C14:1 with a CMC value of >300 μM and C16:0 or C18:0, which have critical micellar temperatures (CMT) of 45°C and 50°C (Small, 1990) respectively, do not inhibit the GAP stimulated p21 GTPase.

Inhibition of the GAP stimulated p21 GTPase by various negatively charged lipids in mixed micelles

In order to establish whether negatively charged lipids in general, provided they are presented in a micellar structure, inhibit the GAP stimulated p21 GTPase, and to compare their relative efficacies as inhibitors, we have incorporated unsaturated and saturated fatty acids as well as various phospholipids and synthetic lipids into mixed Triton X-100 micelles (Hannun *et al.*, 1985; Newton and Koshland, 1989); Triton X-100 micelles are formed at a concentration of

Table I. Comparison of the critical micellar concentration (CMC) values of unsaturated fatty acids and their inhibitory efficacies (IC_{50}) for the GAP stimulated p21 GTPase

Fatty acid	CMC (μM)[a]	IC_{50} (μM)[b]
C14:1	>300	>200
C16:1	100	100
C18:1	ND	50
C18:2	60	70
C18:3	150	150
C20:4	60	60
C22:6	60	50

[a]CMC values were determined by fluorescence spectroscopy using fluorescent indicator molecules.
[b]IC_{50} values were determined by measuring the inhibition of the porcine GAP stimulated p21 GTPase.

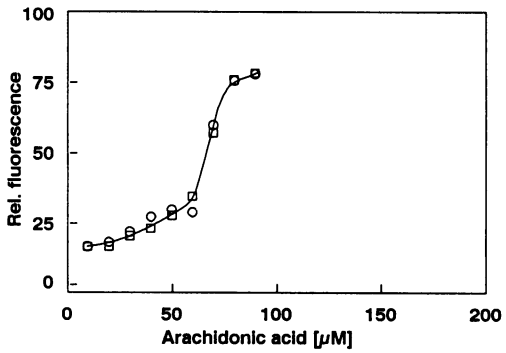

Fig. 2. Determination of the CMC value for arachidonic acid. Increasing amounts of arachidonic acid were incubated with 0.25 μM dephenylhexatriene in 20 mM HEPES, pH 7.6. The fluorescence excitation was at 358 nm, fluorescence emission 430 nm. The rise in fluorescence at 60 μM arachidonic acid is indicative of the phase transition from monomeric fatty acids to micellar aggregates. The two symbols denote the results of two independent titrations.

290 μM Triton X-100, have a mol. wt of ~95 kd, contain on average 140 Triton X-100 molecules/micelle and can accommodate up to 15 mol% of other lipids without dramatic change in structure (Robson and Dennis, 1983; Lichtenberg *et al.*, 1983). The presence of Triton X-100 does not allow, however, the measurement of GTPase activity of p21 with the nitrocellulose assay, because this non-ionic detergent at 'concentrations' >60 nmole/cm^2 interferes with p21−GTP binding to the filter (data not shown). The GTPase measurement was therefore carried out by a modified charcoal adsorption assay (Leupold *et al.*, 1983).

The first series of experiments was carried out with full-length human GAP and physiological lipids, viz. C20:4, C18:3, C18:2, C18:1, C18:0, C16:0, phosphatidic acids with the natural composition of saturated and non-saturated fatty acyl residues, phosphatidylinositol, phosphatidylinositol phosphates (PIP, PIP_2), phosphatidylserine and phosphatidylethanolamine, as well as the rare or non-physiological lipids C16:1, C14:1, distearoyl phosphatidic acid and cetylalcohol phosphoric acid ester. As shown in Figure 3, under identical experimental conditions nearly all lipids with a negative charge led to a more or less pronounced inhibition of the GAP stimulated p21 GTPase. In Table II the relative inhibitory potencies of all lipids tested are given. It is noteworthy that under the conditions of these experiments unsaturated fatty acids are among the most effective

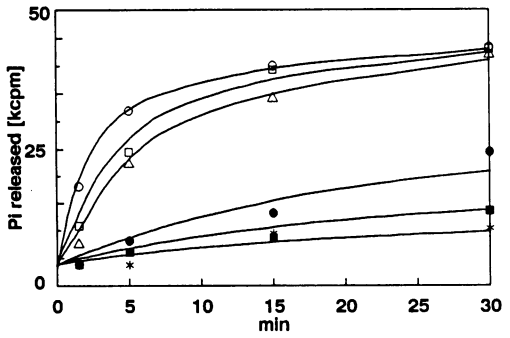

Fig. 3. Kinetics of the GAP stimulated p21 GTPase in the presence of various lipids in mixed micelles. 0.5 μM p21–[γ-^{32}P]GTP, 20 nm human GAP and 14 μM micelles (2 mM Triton X-100, 15 mole% lipid) were incubated in 20 mM HEPES, pH 7.6, at 25°C. The amount of [^{32}P]PO_4^{3-} liberated after defined time intervals was determined by the charcoal adsorption assay. (○) No lipid added; (□) phosphatidic acid; (△) C18:0; (●) C18:1; (■) C20:4; (*) no GAP added.

Table II. Relative inhibitory efficacies of various lipids on the GAP stimulated p21 GTPase in mixed Triton X-100 micelles

Lipid	Inhibition[a] (%)
C20:4, C18:3, C18:2, C18:1, C16:1, C14:1	80–100
Cetylalcohol phosphoric acid ester	40–50
Phosphatidic acid, C20:0, C18:0, C16:0	30–40
Phosphatidylinositol phosphates (PIP, PIP_2)	30
Distearoylphosphatidic acid	20
Phosphatidylinositol, -serine, -ethanolamine	0

[a]The inhibition was determined by measuring the initial rate of GTP hydrolysis with 0.2 μM p21–GTP, 20 nM human GAP and 14 μM mixed micelles (2 mM Triton X-100, 15 mole% lipid). 100% inhibition is defined by the rate of the unstimulated p21 GTPase, 0% inhibition by the rate obtained in the presence of GAP.

inhibitors and that phosphatidylinositol shows no significant inhibition, different from what was reported by Tsai *et al.* (1989a) for pure micelles.

The second series of experiments was carried out with the recombinant C-terminal domain of GAP comprising amino acids 714–1047 and a similar set of lipids as described above for full-length GAP. Essentially the same results were obtained (Figure 4), however, at a lower concentration of the mixed micelles. This may indicate that the C-terminal domain of GAP which has been demonstrated previously to be competent for stimulation of p21 GTPase activity (Marshall *et al.*, 1989) has a lower affinity to its substrate (p21–GTP) (A.Wittinghofer, unpublished) and/or a higher affinity towards the inhibitor.

Sequestration of GAP by pure and mixed micelles

The inhibition of the GAP stimulated p21 GTPase may be due to binding of GAP and/or p21 to the lipid micelles. In order to clarify which protein is the primary target for the inhibition we first analyzed the GAP/p21–GTP reaction in a Michaelis–Menten manner by considering GAP as the enzyme that reacts with p21–GTP as the substrate. As shown in Figure 5 the initial rate of GTP hydrolysis shows a hyperbolic dependence on substrate concentration; in the presence of lipid micelles the initial rate is decreased at all p21–GTP concentrations. This is indicative of an inhibition due to

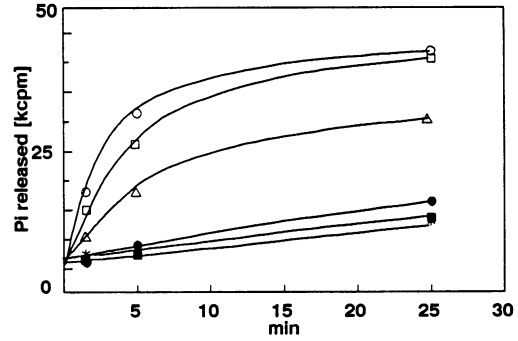

Fig. 4. Kinetics of the p21 GTPase in the presence of the C-terminal domain of GAP various lipids in mixed micelles. 0.5 μM p21–[γ-^{32}P]GTP, 100 nM of the C-terminal domain of GAP and 7 μM micelles (1 mM Triton X-100, 10 mole% lipid) were incubated in 20 mM HEPES pH 7.6 at 25°C. The amount of [^{32}P]PO_4^{3-} liberated after defined time intervals was determined by the charcoal adsorption assay. (○) no lipid added; (□) phosphatidic acid; (△) C18:0; (●) C18:1; (■) C20:4; (*) no C-terminal domain of GAP added.

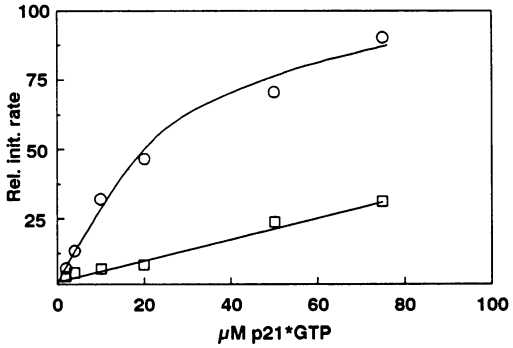

Fig. 5. Dependence of the rate of the GAP stimulated GTP hydrolysis on the concentration of p21–GTP in the absence and presence of lipid micelles. Increasing amounts of p21–[γ-^{32}P]GTP were incubated with 20 nM human GAP and 7 μM pure Triton X-100 micelles (○) or 7 μM mixed micelles (□) (1 mM Triton X-100, 10 mole% arachidonic acid) in 20 mM HEPES, pH 7.6, at 25°C. Initial rates were determined using the charcoal adsorption assay.

binding of the inhibitor to the enzyme (and possibly also the enzyme–substrate complex). These experiments make it likely that GAP is the primary target of the inhibitory lipid micelles.

Binding of the C-terminal domain of GAP to lipid micelles

The interaction of lipid micelles with the C-terminal domain of GAP, of which sufficient amounts were available to carry out physicochemical studies, was demonstrated directly by fluorescence spectroscopy. The intrinsic fluorescence of the C-terminal domain of GAP, which is dominated by the fluorescence of its single tryptophan residue (W885 in full-length GAP) shows a concentration dependent quench down to 60% of its original value upon addition of pure C18:2 (Figure 6) and C20:4 micelles. In contrast, no fluorescence change was observed when p21 was titrated with pure or mixed micelles or when pure micelles were titrated with p21 in the presence of the fluorescent reporter molecules diphenylhexatriene, dimethylanthracene or anthranoyl fatty acids (data not shown).

The results of the fluorescence titrations which demonstrate

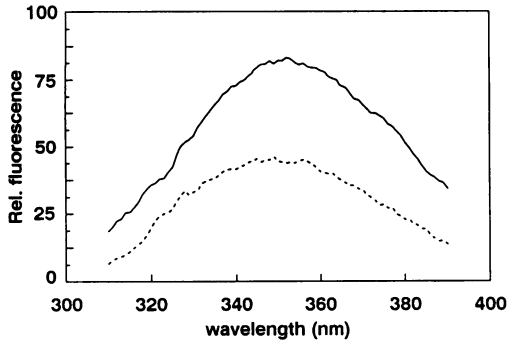

Fig. 6. Fluorescence spectra of the C-terminal domain of GAP in the absence and presence of linoleic acid micelles. The fluorescence emission spectrum of 0.5 μM of the C-terminal domain of GAP was recorded in the absence (−) and presence (----) of 100 μM linoleic acid in 20 mM HEPES, pH 7.6, at 25°C.

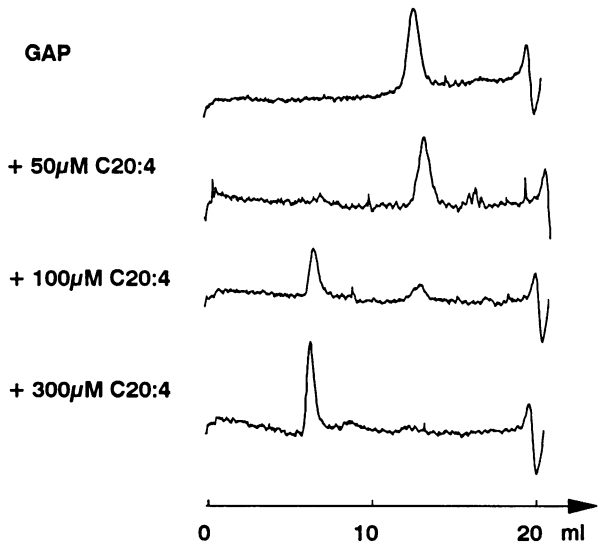

Fig. 7. Analytical gel filtration of the C-terminal domain of GAP in the absence and presence of arachidonic acid micelles. 50 μl samples containing 2 μM C-terminal domain of GAP and 0, 50, 100 and 300 μM arachidonic acid in 20 mM HEPES, pH 7.6, were analyzed by FPLC on a gel filtration column. Individual fractions of the FPLC run were in addition analyzed by TLC and SDS−PAGE to identify the fatty acid and the protein.

that the C-terminal domain of GAP interacts with lipid micelles were confirmed by gel filtration experiments in which the elution volumes of the C-terminal domain of GAP as well as p21 were determined in the absence and presence of lipid micelles. Figure 7 shows four chromatographic runs of GAP on a gel filtration column in the presence of 0, 50, 100 and 300 μM arachidonic acid respectively. At concentrations at and above the CMC value of C20:4 the elution position is shifted to the void volume of the column demonstrating that C20:4 micelles interact with the C-terminal domain of GAP. Similar experiments were also carried out with C18:3, with the same result. In contrast, the elution position of p21 was not shifted by the presence of lipid micelles (data not shown).

Discussion

We show here that various acidic lipids, in particular unsaturated fatty acids, inhibit the GAP stimulated p21 GTPase reaction. The concentration dependence of this inhibition strongly suggests that the formation of micellar structures is a prerequisite for this effect because half maximal inhibition is obtained at the CMC and no inhibition is observed below the CMT. This result is at variance with the conclusion by Tsai *et al.* (1989a), who have argued that the inhibition of the GAP stimulated p21 GTPase is the direct consequence of the molecular structure of the lipids rather than the indirect consequence of their state of aggregation. On the other hand our conclusion offers a straightforward explanation for the results of Tsai *et al.* (1989a) who have measured different potencies of various lipids in inhibiting the p21−GAP interaction: those lipids which have high CMC or CMT values show no or a weak inhibitory effect, while lipids that form micellar structures are potent inhibitors.

Although the aggregated state of acidic lipids is the prerequisite for the inhibition of the GAP stimulated p21 GTPase, not all lipid micelles are equally effective. This is not surprising, because the detailed structure and concentration of lipid aggregates (at a given monomer concentration) are different for all lipids. To assess the inhibitory effect of lipids a standardization is mandatory. This was achieved by experiments with mixed micelles which are obtained by incorporation of equivalent molar amounts of various lipids into Triton X-100 micelles. The results of these experiments show that unsaturated fatty acids are more potent than phosphatidic acids and saturated fatty acids. Neutral phospholipids proved to be inactive in these experiments, while the synthetic cetylalcohol phosphate was active, confirming the conclusion that an acidic function is essential for the inhibitory effect. The different inhibitory potencies of micelles of various acidic lipids presumably is due to the fact that the formation and presentation of a particular flexible array of negative charges on a micellar surface is influenced by the molecular structure of the lipid. This interpretation of our data may also explain the finding of Tsai *et al.* (1989b) that different lipids have different effects on the interaction of Ha-ras, R-ras and Rho proteins with their respective GTPase activating proteins, because a given array of negative charges may be more or less suitable for binding of the protein whose sequestration is responsible for the inhibition of the GTPase activity.

GTPase experiments with varying amounts of p21 at constant concentrations of GAP and lipids indicate that GAP is bound by the lipid micelles, not, however, p21. This is also demonstrated by titration of GAP (C-terminal domain) with lipid micelles which resulted in a quench of the intrinsic fluorescence of GAP. Such effects were not observed with p21. The most direct evidence for an interaction of GAP with lipid micelles is furnished by gel filtration experiments in which the co-elution of a complex of GAP and lipid micelles is demonstrated. These results taken together constitute unequivocal evidence for GAP being the target of lipid micelle mediated inhibition of the GAP stimulated p21 GTPase.

While it is clear that, *in vitro*, the GAP stimulated p21 GTPase can be inhibited by acidic lipids due to binding of GAP via its C-terminal domain to micellar structures, it remains to be demonstrated whether this sequestration is of physiological importance, in particular because it requires a high local concentration of acidic lipids to be effective. *In vivo* these high concentrations are unlikely to be reached in the cytosolic compartment; they could be reached, however, within the membrane. Thus our finding that the

embedding of acidic lipids in micellar structures is essential for their inhibitory activity lends credence to the hypothesis that the sequestration of GAP to patches of the plasma membrane containing acidic lipids may serve to regulate p21. It must be emphasized that the regulation of the p21 activity in order to be fail-safe most likely is manifold; the sequestration of GAP may be one mechanism among others which are operating synergistically to control the activity of p21.

Materials and methods

Lipids

All lipids were purchased from Sigma (München), with the exception of docosahexaenoic acid which was obtained from Roth (Karlsruhe), and cetylalcohol phosphoric acid which was a kind gift from Dr Eibl (Göttingen).

Ha-ras p21

p21 was overexpressed in and isolated from *E.coli* as described (Tucker *et al.*, 1986). p21 was depleted of its bound nucleotide by extensive dialysis (Ferguson *et al.*, 1986) against 1.5 M $(NH_4)_2SO_4$ in 50 mM Tris–HCl, pH 7.6, and stored at −80°C at a concentration of 200 μM. HPLC analyses showed that ~75% of the bound nucleotides were removed by this treatment.

GAP

Three different GAP preparations were used. (i) Porcine GAP was enriched (Gibbs *et al.*, 1988) from a high speed supernatant of homogenized pig brain using anion exchange chromatography on DEAE CL 6B. This preparation, ~30-fold enriched in GAP, was stored in the presence of 1 mM NaN_3 and 10% v/v glycerol at 4°C. (ii) Human GAP was isolated as described from S_f9 cells infected with recombinant baculovirus (Halenbeck *et al.*, 1991) and stored at 4°C. (iii) A truncated version of human GAP comprising amino acids 714–1047 was isolated from *E.coli* cells transformed with the plasmid ptrc99c which carries the truncated human *GAP* gene under the control of the *tac* promoter. The isolation procedure followed a protocol worked out by J.E.Scheffler (personal communication). After lysis of 100 g *E.coli* cells (wet paste) with 1 mg/ml lysozyme in 50 mM Tris–HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA, 1 mM DTE and 0.4 mM PMSF (standard buffer), incubation with 0.075% w/v deoxycholate for 30 min and with 0.01 mg/ml DNase I for another 30 min, the suspension was centrifuged for 1 h at 30 000 *g*. The supernatant was loaded onto a 5 × 35 cm DEAE Sepharose CL 6B column. Gradient elution was carried out with 2 × 2 l of 0–200 mM NaCl in standard buffer. Fractions containing GAP as shown by SDS–PAGE were pooled, concentrated by ultrafiltration using Amicon YM 10 membranes and then loaded onto a 5 × 185 cm Sephadex G-75 column. Elution was carried out with 20 mM Tris–HCl, pH 8.5, 1 mM EDTA, 1 mM DTE. Fractions containing pure GAP were pooled, concentrated and stored at a concentration of ~10 mg/ml at 4°C or −80°C.

GTPase assays

The GTPase activity of p21 was determined by nitrocellulose filtration of p21–[γ-^{32}P]GTP (Gibbs *et al.*, 1988) or adsorption of [^{32}P]PO_4^{3-} to activated charcoal (Leupold *et al.*, 1983).

Preparation of pure and mixed micelles

For the preparation of pure micelles the required amounts of methanolic stock solutions of the lipids were dried *in vacuo* and dissolved in 20 mM HEPES, pH 7.6, by occasional agitation at 25°C for 10 min. Mixed micelles were prepared by solubilization of dried lipids in a buffered 5 mM Triton X-solution. For saturated fatty acids this preparation was carried out at 56°C.

Fluorimetric determination of the critical micellar concentrations of fatty acids

CMC values for fatty acids were determined by fluorescence spectroscopy (Chattopadhyay and London, 1984) using the fluorescent probe diphenylhexatriene (DPH). A DPH solution (2 μl) in tetrahydrofurane was added in the dark to the lipid solution in 20 mM HEPES, pH 7.6, to give a final concentration of 0.25 μM. Fluorescence measurements were carried out at 25°C in a Schoeffel RRS 1000 spectrofluorimeter. Excitation was at 358 nm, emission spectra were recorded and the fluorescence intensity, measured at the emission maximum, was used for data evaluation.

Fluorescence titrations of the C-terminal domain of GAP with lipid micelles

0.5 μM of the C-terminal domain of GAP was titrated with concentrated solutions of lipid micelles in 20 mM HEPES, pH 7.6, at 25°C. Fluorescence emission spectra were recorded in a Schoeffel RRS 1000 spectrofluorimeter at 300–400 nm with excitation at 280 nm.

Analytical gel filtration of the C-terminal domain of GAP in the absence and presence of lipids

Gel filtration experiments were carried out on a Superose 12 HR 10/30 column (Pharmacia) using the Pharmacia FPLC system. A 50 μl solution of 2 μM of the C-terminal domain of GAP or p21 in 20 mM HEPES, pH 7.6, was incubated in the absence or presence of 50, 100 and 300 μM of arachidonic acid and linoleic acid respectively for 10 min and then loaded onto the gel filtration column equilibrated with 50 mM Tris–HCl, pH 7.6. The flow rate was 0.5 ml/min. Absorbance was measured at 220 nm.

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