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The GTPase stimulatory activities of the neurofibromatosis type 1 and the yeast IRA2 proteins are inhibited by arachidonic acid

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Three proteins, GTPase activating protein (GAP), neurofibromatosis 1 (NF1) and the yeast inhibitory regulator of the RAS-cAMP pathway (IRA2), have the ability to stimulate the GTPase activity of Ras proteins from higher animals or yeast. Previous studies indicate that certain lipids are able to inhibit this activity associated with the mammalian GAP protein. Inhibition of GAP would be expected to biologically activate Ras protein. In these studies arachidonic acid is shown also to inhibit the activity of the catalytic fragments of the other two proteins, mammalian NF1 and the yeast IRA2 proteins. In addition, phosphatidic acid (containing arachidonic and stearic acid) was inhibitory for the catalytic fragment of NF1 protein, but did not inhibit the catalytic fragments of GAP or IRA2 proteins. These observations emphasize the biochemical similarity of these proteins and provide support for the suggestion that lipids might play an important role in their biological control, and therefore also in the control of Ras activity and cellular proliferation.

Key words: arachidonic acid/IRA2/neurofibromatosis 1/ proliferation/Ras

Introduction

Cellular Ras proteins play a critical role in the control of proliferation in higher eukaryotic cells. When *ras* activity is removed from mammalian cells by microinjection of a neutralizing antibody (Mulcahy *et al.*, 1985) or dominant inhibitory mutants (Stacey *et al.* 1991) the cells are unable to proliferate. In addition, many naturally occurring tumors contain activating mutations in members of this gene family (Bos, 1989). The control of cellular Ras activity is not well understood, but clearly involves the GTPase activating protein (GAP). GAP is a cytosolic protein that catalyzes conversion of the biologically active, GTP-bound form of Ras to the inactive GDP-bound form (Trahey and McCormick, 1987) and thereby biologically inactivates Ras (Zhang *et al.*, 1990). Oncogenic mutants of Ras escape down-regulation by GAP and thus remain in the GTP-bound form constitutively (Trahey and McCormick, 1987).

The bovine GAP protein, 1044 amino acids long, has at least two domains, with the carboxy-terminal 343 amino acids alone able to stimulate Ras GTPase activity (Marshall *et al.*, 1989). The amino-terminal noncatalytic domain contains two SH2 (*src* homology) domains related to regulatory sequences of some nonreceptor tyrosine kinases, phospholipase Cγ and the *crk* oncogene (Pawson, 1988). In addition to a role in down-regulating Ras activity, GAP has been implicated as an effector of Ras function (Hall, 1990; Farnsworth *et al.*, 1991) and also as a component of signaling complexes that include activated PDGF receptors (Molloy *et al.*, 1989; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990) and other tyrosine kinase substrates (Ellis *et al.*, 1990). Therefore, GAP may play a role in both Ras and tyrosine kinase signaling pathways.

Recently, a new Ras GTPase activating protein was identified as the product of the gene containing exon-based mutations isolated from patients suffering from one of the most common autosomal dominant disorders, neurofibromatosis type 1 (NF1) (Cawthon *et al.*, 1990; Wallace *et al.*, 1990; Viskochil *et al.*, 1990). Mutations in the *NF1* gene lead to the formation of neurofibromas and alterations in a variety of tissues including increased incidence of some types of spontaneous malignancies (Cohen and Rothner, 1989). The NF1 gene is predicted to encode a protein of at least 2485 amino acids, which contains a region of ~350 amino acids that shows 30% sequence homology to the catalytic domain of mammalian GAP and yeast IRA proteins (Xu *et al.*, 1990a). Clear evidence that the NF1 protein can act as a GTPase activating protein for Ras was obtained when the GAP-related region of NF1 stimulated GTP hydrolysis of the Ras, and substituted for IRA proteins in yeast (Xu *et al.*, 1990b; Martin *et al.*, 1990; Ballester *et al.*, 1990).

In addition to NF1 and GAP, the yeast IRA1 and IRA2 proteins have been shown to regulate the activity of yeast RAS proteins. Both proteins function to down-regulate RAS activity by stimulating the GTPase activity of RAS1 and RAS2 proteins (Tanaka *et al.*, 1990). Moreover, an IRA2 peptide spanning the GAP homology domain stimulates the GTPase activity of yeast RAS2 *in vitro* (Tanaka *et al.*, 1991). The IRA2 protein of 2938 amino acids is homologous to the *NF1* gene not only in the catalytic domain, but contains additional similarities in the regions on either side of the catalytic domain (Xu *et al.*, 1990a).

The GTP binding and hydrolyzing activity of Ras proteins initially suggested that they, like other G-proteins, might be involved in an intracellular signaling mechanism. Unlike many GTP binding-proteins, however, no physical interactions with other protein subunits or with transmembrane receptor molecules, which might help explain the mechanism of the control of Ras activity, have been identified. While it is clear that GAP functions to regulate Ras activity, it is

not clear how GAP itself might be regulated. Recent studies show that GAP is phosphorylated by and associates with growth factor receptor tyrosine kinases (Ellis *et al.*, 1990; Molloy *et al.*, 1989). Yet the proportion of the GAP molecules associated with the receptors is small, and the biochemical consequences of phosphorylation have yet to be demonstrated. The profound sensitivity of lipid-induced mitogenesis to inhibition by injection of anti-ras antibody (Yu *et al.*, 1988), suggested some time ago that lipids might be involved in the control of ras activity. Several recent studies support this hypothesis. Lipids produced soon after mitogenic treatment are able to inhibit the ability of GAP to stimulate the GTPase activity of Ras, and are able to physically bind the GAP molecule (Yu *et al.*, 1990; Tsai *et al.*, 1991).

When it was shown that both NF1 and IRA2 stimulate the GTPase activity of their respective Ras proteins, it became of interest to determine if they are also sensitive to inhibition by lipids. This would strengthen the possibility that lipids play a biologically important role in the control of Ras activity by demonstrating that lipid sensitivity is a common characteristic of GTPase stimulatory proteins. The demonstration here that the catalytic domains of both NF1 and IRA2 proteins are also inhibited by arachidonic acid serves as a further demonstration of the biochemical similarity between these three proteins. Partial inhibition of NF1 protein by phosphatidic acid (containing arachidonic and stearic acid) distinguishes NF1 protein from the other two GTPase activating proteins, emphasizing the individuality of the activity of the three proteins.

Results

Inhibition of the NF1 catalytic fragment by arachidonic acid

Due to its size and the uncertainty concerning its amino-terminus, the NF1 protein has been expressed only as a catalytic, GAP-homologous fragment. In order to relate NF1 activity to that of GAP, therefore, we used a bacterial expression vector for the catalytic fragment of GAP. For comparison with the catalytic fragment of NF1 (from amino acid 840–1204; termed NF1 hereafter), a fragment of GAP from amino acid 702 to the carboxy-terminus (amino acid 1044; termed GAP 702 hereafter) was utilized (Marshall *et al.*, 1989). Both proteins were prepared from bacterial expression vectors (Marshall *et al.*, 1989; Tanaka *et al.*, 1991). The NF1 catalytic fragment was purified as a fusion protein with glutathione-S-transferase (GST). Earlier it was shown that GST has no influence on GTPase activity of Ras protein (Xu *et al.*, 1990b). An accurate comparison requires that the same amount of GTPase stimulatory activity should be used in assays of each protein. A dose-response analysis was, therefore, performed first to determine the amount of each protein required to induce hydrolysis of Ras to between 80 and 90% in a 15 min incubation. It is important to control the GTPase activating activity in the assays to ensure that the extent of GTP hydrolysis by Ras would be responsive to subtle changes in the activity of the stimulatory protein, and to ensure that the results with different GTPase stimulatory proteins can be compared.

When the amount of GTPase activating activity for GAP 702 was normalized to that of NF1, the ability of lipids to inhibit each protein was determined. A time course comparison between NF1 and the GAP 702, using a filter binding assay for Ras GTPase activity, was performed in the presence of three different lipids, each at a concentration of 100 μg/ml. For the filter binding assay, purified, bacterially produced Ras protein was allowed to bind γ-labeled GTP prior to incubation with GTPase activating proteins. After incubation the amount of unhydrolyzed, Ras-bound GTP was determined by retention of proteins within the reaction mixture on nitrocellulose filters. Hydrolysis of bound GTP would result in loss of the labeled phosphate

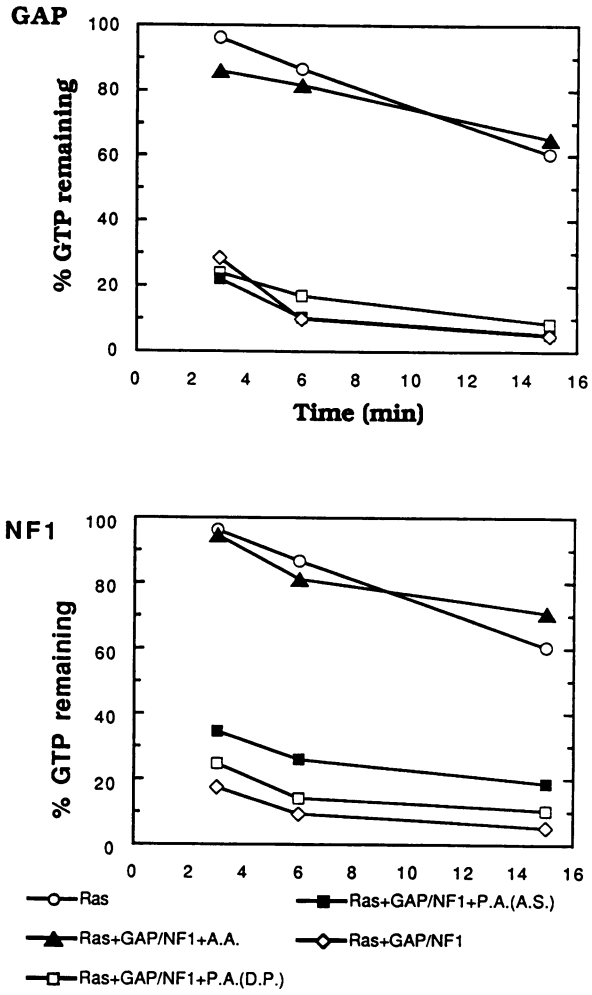

Fig. 1. Time course of inhibition of NF1 and GAP 702 by lipids. The bacterially synthesized, carboxy-terminal fragment of GAP (top panel) or the catalytic fragment of NF1 (bottom panel) were assayed for their ability to stimulate the GTPase activity of Ras protein in the presence of 100 μg/ml of the indicated lipids. The point indicating 100% GTP remaining refers to the amount of label associated with Ras without incubation and typically represented an average of 35 000 c.p.m. Background readings were <2% this value. All experiments presented above were performed simultaneously and repeated several times with similar results. It is apparent that the arachidonic acid (AA, triangles) inhibited both GAP 702 and NF1 completely, as indicated by the fact that the amount of GTP remaining was essentially the same as seen in reactions containing Ras without added GAP. Partial inhibition of NF1 was seen with phosphatidic acid containing arachidonic and stearic acids [P.A. (A.S.), solid squares] as indicated by comparison to reactions containing Ras and NF1 in the absence of lipids (diamond). This lipid was never seen to inhibit GAP 702. No inhibition was seen with the phosphatidic acid containing only palmitic acid [P.A. (D.P.), open squares]. Very similar results were obtained when bovine hemoglobin was used in the place of BSA as a carrier protein in the GTPase reactions.

(see Materials and methods). It was clear from the results that the stimulation of GTP hydrolysis by NF1 was inhibited by arachidonic acid (AA) as efficiently as was GAP 702. The AA at 100 μg/ml inhibited the GTPase stimulatory activity of both proteins essentially completely (Figure 1). The endogenous rate of GTPase activity of Ras protein was not altered by the tested lipids (not shown).

To determine the relative efficiency of the inhibition by AA, a dose response analysis of lipid inhibition of NF1 and GAP 702 was next performed using the filter binding and immunoprecipitation assays of GTP hydrolysis by Ras. For the immunoprecipitation assays Ras binds α-labeled GTP prior to incubation with the GTPase activating protein. After incubation the Ras protein and associated nucleotide is immunoprecipitated, the nucleotide released and resolved on a TLC plate into GTP and GDP. The ratio of these two species indicates the exent of GTP hydrolysis. For determinations utilizing the immunoprecipitation assay, one time point was taken after a 15 min incubation, at which time hydrolysis of GTP was ~90% complete with both NF1 and

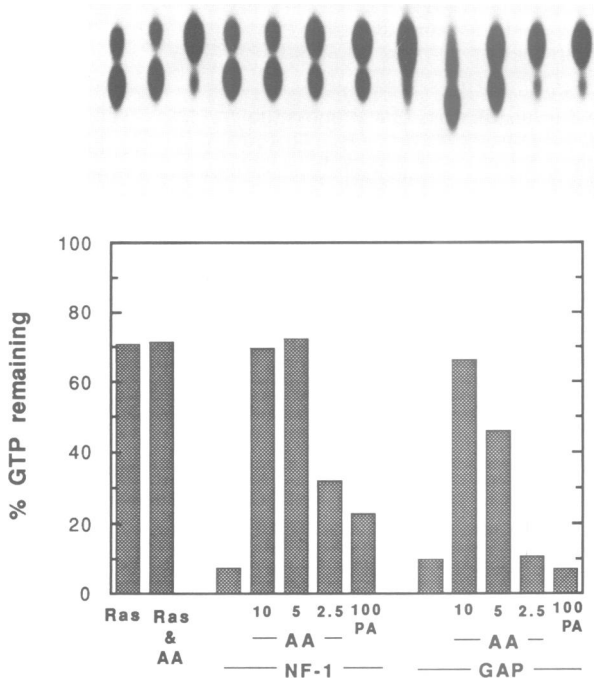

Fig. 2. Dose-response of lipid inhibition. This immunoprecipitation assay was performed to determine the lowest amounts of arachidonic acid needed to inhibit GAP 702 or the catalytic fragment of NF1. Reactions were performed simultaneously in the presence of the indicated GTPase stimulatory protein together with the indicated amount of lipid (μg/ml). Ras protein was allowed to associate with α-labeled GTP prior to incubation for 15 min at 30°C. Ras protein in the reaction was immunoprecipitated and bound nucleotides were resolved on a TLC plate into GTP (lower spots) and GDP (upper spots). A photograph of the autoradiogram of the resulting TLC plate is included (top panel) together with a quantification of the percentage of GTP remaining associated with Ras at the end of the incubation (bottom panel). The order of samples in the autoradiogram is the same as those shown below. Complete inhibition of the added GTPase stimulatory protein would result in GTP percentages similar to that seen with Ras alone in the reaction mixture. Without inhibition, the percentage of GTP remaining associated with Ras would be the same as the reaction in the presence of the stimulatory protein but without indicated lipid addition. Partial inhibition of GTPase stimulatory activity was seen for NF1 with 2.5 μg/ml AA; and for GAP 702 at 5 μg/ml. The addition of 100 μg/ml phosphatidic acid (β-arachidonoyl-γ-stearoyl) inhibited NF1 partially, but was not inhibitory for GAP 702. As a control, the addition of AA at 100 μg/ml did not significantly alter the GTPase activity of Ras alone.

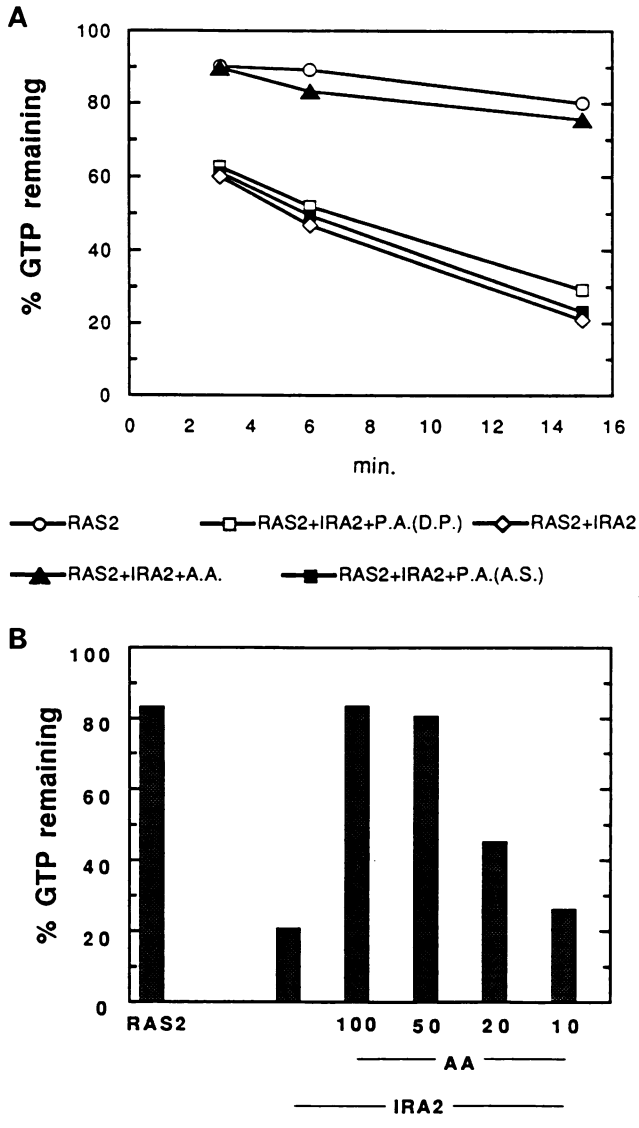

Fig. 3. Inhibition of the IRA2 activity by arachidonic acid. (**A**) A time course of GTPase activity was performed with the catalytic fragment of IRA2 protein similarly to that presented in Figure 1. The indicated lipids were added at 100 μg/ml. The endogenous rate of RAS2 GTPase activity (indicated by reactions with RAS2 alone, circles) was low. The addition of IRA2 protein (diamonds) increased the hydrolysis to almost 80% after 15 min. Addition of phosphatidic acids (open and closed squares) did not affect the stimulation of GTPase by IRA2 protein. The arachidonic acid (triangles), however reduced the rate of GTPase in the presence of IRA2 to near that observed in the absence of IRA2. (**B**) A dose-response of lipid inhibition was next performed for IRA2 stimulation of yeast RAS2 protein (similar to the determination in Figure 1B; except that filter binding instead of immunoprecipitation assays were performed). The indicated amounts of arachidonic acid were added to the reactions which were incubated for 15 min prior to filter binding. It is clear that partial inhibition of GTPase stimulation was seen with 20 μg/ml AA, while 10 μg/ml give little detectable inhibition. Complete inhibition, as indicated by comparison of %GTP remaining with the reaction containing only RAS, was observed with both 100 and 50 μg/ml.

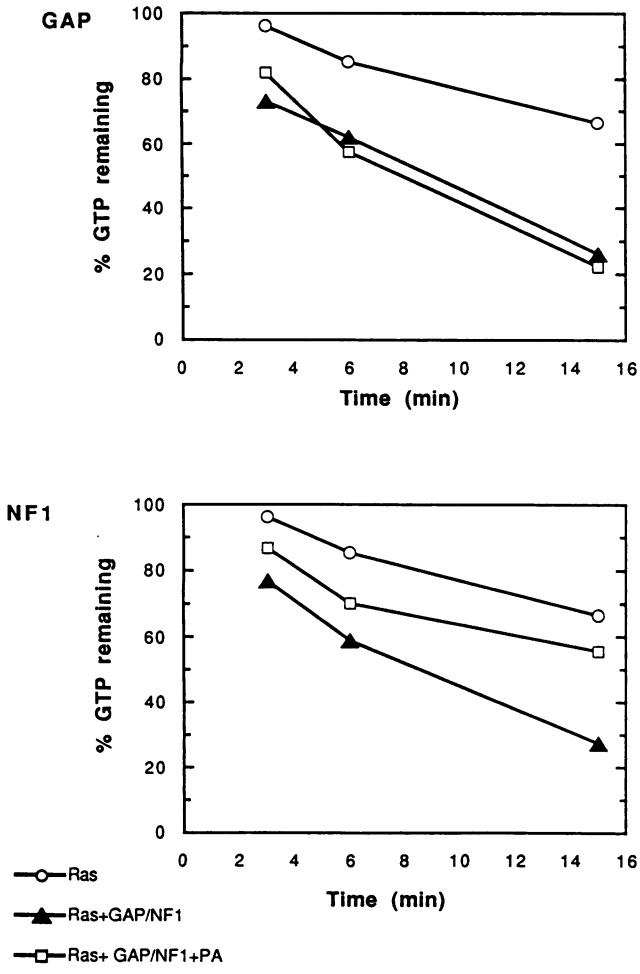

Fig. 4. Time course of inhibition of NF1 and GAP 702 by phosphatidic acid (β-arachydonoyl-γ-stearoyl) (PA). Data presented above with GAP 702 (top panel) and NF1 (bottom panel) were performed simultaneously as described in Figure 1. The amount of NF1 and GAP in these reactions was four times smaller than in the assays described in Figure 1. Under such conditions the GTPase activity of NF1 was inhibited ~50%, significantly better than in the previous experiments. However, no inhibition of GAP 702 with this lipid was seen.

GAP 702. In the presence of 10 or 5 μg/ml AA, inhibition of NF1 activity was essentially complete. With 2.5 μg/ml the activity of NF1 was reduced by 40% (while lower concentrations exhibited no inhibition). GAP 702 was inhibited completely only with 10 μg/ml or more AA. Inhibition of GAP 702 was reduced to 60% with 5 μg/ml, while 2.5 μg/ml AA was not inhibitory (Figure 2). These results were repeated with the filter binding assay at earlier time points (the third and sixth minute) with the same results; the NF1 was always ~2-fold more sensitive to inhibition with AA (not shown). Inhibition with AA was highly consistent between different determinations. While subtle variations were at times seen between the inhibitory activity of different preparations of AA, this inhibitory activity was invariably 2-fold more effective against NF1 than GAP 702.

Inhibition of IRA2 by arachidonic acid

In addition to NF1 and GAP, the yeast IRA1 and IRA2 proteins have been shown to regulate the activity of yeast RAS proteins. We next tested the possibility that the IRA2 protein might also be inhibited by lipids. As above, the catalytic domain of IRA2 (amino acids 1665–2025; hereafter termed IRA2) was expressed in bacteria (Tanaka *et al.*, 1991) and assayed in the presence of lipids. IRA2 was assayed with a different target protein (yeast RAS2) than GAP 702 or NF1. The stimulation of RAS2 hydrolysis by IRA2 was not as efficient as described above using mammalian proteins, and was only between 70 and 80% after 15 min. Nevertheless, it was apparent that AA at 100 μg/ml completely inhibited this activity at all times tested (Figure 3a). In these experiments with IRA2, as well as in the work described above with NF1, the phosphatidic acid (dipalmitoyl) exhibited a slight inhibition of GTPase activity. The extent of this inhibition, however, appears to be too low to be of significance. Finally, a dose-response analysis of IRA2 inhibition by AA was performed using the filter binding assay (Figure 3b). Inhibition of the catalytic fragment of IRA2 was essentially complete in the presence of 100 and 50 μg/ml AA, but with 20 μg/ml the lipid inhibited catalytic activity only ~40%. Little inhibition was observed with 10 μg/ml AA or less (Figure 3b).

Inhibition of NF1 by phosphatidic acid (β-arachidonyl-γ-stearoyl)

While all three GTPase activating proteins were inhibited by arachidonic acid, in the case of NF1, phosphatidic acid (β-arachidonyl-γ-stearoyl) was inhibitory to a limited extent. This observation was repeated a number of times with the filter binding and also the immunoprecipitation assay of GTPase activity (see Figures 1 and 2). Analyses with one type of assay were routinely verified with the other to ensure the accuracy of the results. Inhibition by phosphatidic acid was never more than 30%, and in some cases little inhibition was observed. In no case, however, was there any evidence that this lipid had inhibited GAP 702; nor was there any indication that dipalmitoyl phosphatidic acid was inhibitory. To this extent, therefore, there is a clear distinction in the profile of lipid inhibition between the two proteins.

Since inhibition of NF1 by phosphatidic acid (β-arachidonyl-γ-stearoyl) was weak in comparison to inhibition by AA, the assays were repeated with less GTPase stimulatory protein in order to monitor the reaction in its more linear range. In this case, the extent of GTP hydrolysis after 15 min incubation was only ~70%, compared to hydrolysis near 90% in studies discussed above. Once again, care was taken to ensure that GTP hydrolysis in the presence of NF1 was roughly comparable to that in the presence of GAP 702. In these assays the inhibition of NF1 by phosphatidic acid was much more evident, being ~50%, i.e. ~20% higher than in the previous experiments (Figure 4). In no case, however, was there any evidence of inhibition of GAP 702 by phosphatidic acid.

The apparent selective inhibition of NF1 by phosphatidic acid (β-arachidonyl-γ-steoryl) compared to GAP 702 might indicate a difference in their fundamental biochemical properties and perhaps their means of biological control. It was therefore critical to rule out the possibility that free arachidonic acid contaminating the phosphatidic acid preparation, rather than the phosphatidic acid itself, might have been responsible for the inhibition observed. As demonstrated below, NF1 is slightly more sensitive to arachidonic acid than is GAP 702. Therefore, we separated phosphatidic acid by thin layer chromatography (TLC) using a solvent system in which phosphatidic acid remains near

the origin and AA has R_f value of ~0.6. The resulting TLC plate was then divided into sections, one near the origin of the plate and the other corresponding to AA (as determined from the parallel plate stained for lipid standards). Lipids were extracted from each section and the extracts tested for the ability to inhibit GAP 702 and NF1 GTPase activity (for procedure see, Yu *et al.*, 1990). The inhibitory activity was extractable only from the section containing phosphatidic acid and it was inhibitory only to NF1 but not to GAP 702 (not shown). This confirmed our confidence that the inhibitory effect was not a result of hydrolysis of AA from phosphatidic acid. The significance of the phosphatidic acid (β-arachidonyl-γ-stearoyl) inhibition is uncertain due to the variation in results with different preparations of this lipid, and the limited extent of inhibition even at relatively high concentration. It is possible that phosphatidic acid mimics poorly a more efficient inhibitor of NF1 (Corven *et al.*, 1989).

Discussion

Changes in lipid metabolism are among the earliest and most pronounced metabolic alterations induced by mitogenic stimulation. A direct role of lipid metabolism in the control of proliferation was suggested by injection of anti-ras antibody. In these studies it was found that mitogenesis, induced by mitogenic agents related to lipids, was unusually dependent upon cellular Ras activity (Yu *et al.*, 1988). This observation suggested that lipids might directly control the activity of cellular Ras proteins. Subsequent studies have provided support for this possibility. First, the ability of GAP to stimulate the GTPase activity of cellular Ras, and thereby down-regulate its biological activity, is inhibited by several lipids whose metabolism is characteristically altered by mitogenic treatments (Tsai *et al.*, 1989b); including phosphatidic acid (β-arachidonoyl-γ-stearoyl), arachidonic acid, and phosphatidyl inositol monophosphate. Lipids were also able to inhibit the activity of distinct GTPase activating proteins in their interaction with two Ras-related proteins (Tsai *et al.*, 1989a). More recent studies indicate that lipids with the ability to inhibit GAP activity are produced within 1−5 min of mitogenic stimulation of NIH3T3 cells, but that these lipids are formed only in subconfluent cultures which are able to actively respond to the mitogenic treatments (Yu *et al.*, 1990). Finally, it is clear that the GAP molecule is able to physically associate with lipids, but only those lipids which were previously shown to inhibit GAP activity, and only in the presence of divalent cations (Tsai *et al.*, 1991).

Despite the genetic and biochemical evidence cited above, there is no direct evidence relating lipids to the normal biological control of GAP or of Ras activity. With the demonstration that NF1 is able to stimulate the GTPase activity of Ras, however, the opportunity to gain such evidence was presented. It is clear that the NF1 gene is involved in proliferative control as evidenced by the biological consequences of its mutation (Riccardi, 1981). While GAP and NF1 share the ability to stimulate GTPase activity, their catalytic domain sequences are not identical (30% homology); and outside this domain their sequences diverge (Xu *et al.*, 1990a). It is likely, under these circumstances, that only essential biochemical properties would be conserved between the two molecules. The fact that each is inhibited by a similar set of lipids in a highly analogous way argues strongly that this lipid inhibition is critical for the activity of both proteins. The subtle differences in lipid inhibition observed between the two proteins indicate possible subtle distinctions in their biological functions. When the inhibition by lipids of the yeast IRA protein is considered, the evidence that lipids are part of the normal control of cellular Ras activity is strengthened further.

Due to its size and the lack of information concerning its amino-terminus, it is possible at present to study only the catalytic fragment of NF1. For this reason, its activity was carefully compared to a catalytic fragment of GAP, GAP 702. Both proteins, along with the catalytic fragment of IRA2, were inhibited by arachidonic acid. The NF1 protein was ~2-fold more sensitive to inhibition by this fatty acid than was GAP 702, with IRA the least sensitive. The variations in these sensitivities might reflect sub-optimal assay conditions for one or all of these proteins, since all assay conditions were consistent throughout this work. Alternatively, the biological role of lipid inhibition of the individual proteins might dictate differences in their sensitivities to inhibition by a given lipid. With phosphatidic acid there were qualitative distinctions in lipid inhibition between the three proteins: only NF1 was inhibited by this lipid. This fact emphasizes the individuality of the activity of the three proteins. It is likely, however, that lipids related to phosphatidic acids but with greater inhibitory activity exist (Corven *et al.*, 1989). If these were identified, even greater distinctions might have been observed between the three proteins.

Even though full-length NF1 is not available for comparison to its catalytic fragment, it is possible to compare the activity of the catalytic fragment of GAP, GAP 702, to the activity of other GAP fragments of varying size and to the full-length molecule. By relating the activity of catalytic GAP fragments to the activity of the intact protein it might be possible to predict the relationship between the fragment of NF1 studied here and the full-length protein. A variety of amino-terminal deletions of the GAP molecule from 128 to 702 amino acids have been compared for lipid inhibition characteristics (unpublished data). It was found that there was no distinction between GAP 702 and any of the larger fragments either in the types of inhibitory lipids or the extents of inhibition. There were, however, distinctions between the catalytic GAP fragments and full-length GAP; and even between full-length bacterially synthesized and cellular GAP molecules. While arachidonic acid efficiently inhibited the fragments, the full-length molecule was not completely inhibited by this lipid (at 100 μg/ml). Like the fragments, full-length GAP produced in bacterial cells was not inhibited by phosphatidic acid. For comparison, the GAP produced within living cells is inhibited by phosphatidic acid (Tsai *et al.*, 1989b). Further studies are underway to determine the molecular basis for the distinctions between bacterial and cellular GAP molecules. These studies indicate that while the general properties of lipid inhibition are retained by catalytic fragments of a GTPase activating protein, there are subtle differences between them and the full-length molecule. We conclude, therefore, that while studies with NF1 catalytic fragments are likely to provide general information concerning lipid inhibition characteristics, such studies must be verified and extended in experiments with the full-length molecule.

Recent studies have related the inhibition of GAP by lipids

to their ability to form micelles (Serth *et al.*, 1991). In the work reported here, however, the catalytic fragments of all three proteins tested were inhibited by concentrations of arachidonic acid well below the concentration required for micelle formation. While the association of full-length GAP with lipid micelles might be an important aspect of lipid inhibition, this is apparently not the only factor involved in lipid inhibition.

These results support the concept that lipids play an important role in the control of Ras activity (Stacey *et al.*, 1988). As stated previously, there are a variety of experiments which support this suggestion in mammalian cells. The possibility that lipids also control the activity of RAS proteins in yeast cells is clearly suggested by the observation that the IRA2 catalytic fragment is inhibited by AA. Taken together, these results emphasize the possibility that lipids play a fundamental role in the regulation of Ras proteins in all eukaryotic cells, and provide a basis for further studies of the respective full-length GTPase stimulating proteins. The fact that arachidonic acid is inhibitory for each of these GTPase stimulatory proteins is interesting in view of the release of this lipid in many physiological circumstances, and the fact that it is metabolized to a variety of biologically active molecules, some of which appear to be even more inhibitory to GAP than is arachidonic acid itself (Yu *et al.*, 1990). Due to the clinical importance of mutations in the NF1 gene, the possibility that lipids might be involved in its control warrants further study. It will be important to determine if naturally occurring mutations in this gene might alter its interaction with cellular lipids, and thereby its response to biological control mechanisms.

Materials and methods

Partial purification of GAP 702, NF1 and IRA 2 catalytic fragments

Partial purification of GAP 702 was done with a modification of the protocol described by Marshall *et al.*, 1989. Deletion mutant GAP 702 was expressed in *E.coli* strain RR1laci^q by overnight growth in LB medium with 50 μg/ml ampicillin and induced with isopropylthio β-D-galactosidsae (IPTG; final concentration 100 μM). Cells from 4 l of culture were lysed with a French Pressure Cell in 60 ml of buffer containing 50 mM Tris−Cl, pH 7.5, 1 mM EDTA, 5 mM DTT, 200 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml antipain, 1 μg/ml leupeptin and 10 μg/ml benzamidine. All protease inhibitors were purchasd from Sigma. $MnCl_2$ and $CaCl_2$ were added to the lysate to 0.85 and 0.12 mM respectively, which was digested with DNAse I (final concentration 10 μg/ml) for 20 min at 4°C. The lysate was cleared by centrifugation at 15 000 *g* for 20 min at 4°C. The supernatant was applied onto a 2.5 × 30 cm DEAE−Sephacel column (Pharmacia-LKB) and washed until the absorbance reached baseline. Elution was achieved with a 500 ml 0.0−0.45 M NaCl linear gradient at a flow rate of 0.3 ml/min while collecting 4.5 ml fractions. The active fractions (as determined by assay with Ras protein) were pooled and concentrated to 1−2 ml by filtration with Amicon PM-10 membrane in a stirred cell. The concentrated sample was applied to a 1.5 × 50 cm Sephadex G-100 column and 1.0 ml fractions were collected. The active fractions were concentrated as before to 1.5 ml and used in the GTPase assays.

The NF1 catalytic fragment (840−1204) was purified as a fusion protein with glutathione-S-transferase (GST) as described by Xu *et al.* (1990b). GST-NF1 was affinity purified from soluble extracts of *E.coli* cells using glutathione agarose beads (Sigma). Bound protein was eluted into 50 mM Tris−Cl (pH 7.5) and 5 mM reduced glutathione (Sigma). Concentrated protein was used for the GTPase assays.

The catalytic domain of IRA2 (1665−2025) was fused to the C-terminal end of GST and the GST-IRA2 fusion protein was expressed in *E.coli* cells and was affinity purified as described by Tanaka *et al.* (1991).

GTPase assays

Filter binding assay was preformed according to protocol described by Tsai *et al.* (1989a). Briefly, purified, bacterially synthesized Ha-Ras was incubated for 5 min at 30°C with [γ-^{32}P]GTP (30 Ci/mmol) in buffer containing 20 mM Tris−Cl, pH 7.5, 1 mM DTT. GTPase activity was then assayed by incubating the [γ-^{32}P]GTP loaded Ras at 30°C in 60 μl reaction buffer (20 mM Tris−Cl pH 7.5, 1 mM DTT, 5 mM $MgCl_2$), fatty acid-free BSA 50 μg/ml (from Sigma), 0.5 mM unlabeled GTP and 0.24 mg/ml of partially purified GAP 702 or 1.5 μg/ml of more highly purified NF1 were also included. The final concentrations of Ras protein and radiolabeled GTP were 30 nM and 300 nM respectively. At each time point the test samples were put on water/ice bath, filtered on nitrocellulose filter (0.45 μm; Schleicher & Schuell) and washed once with 14 ml of 20 mM Tris buffer (pH 7.5) containing 5 mM $MgCl_2$ and 50 mM NaCl. The amount of [γ-^{32}P]GTP remaining on the filters which reflects the GTPase activity of Ras, was then determined by liquid scintilation counting in 5 ml of CytoScint (ICN Biomedicals, Inc.). To ensure the equal GTPase activity of NF1 and GAP 702 protein preparations, both of them were tested in varying amounts in Ras GTPase assay. The amount of protein preparations that caused about 90% of GTP hydrolysis after 15 min of incubation at 30°C were used in all subsequent experiments. At the same time point Ras protein itself had hydrolyzed ~30% of bound GTP and by monitoring the reaction at the third and sixth minute the observed inhibitions were occurring in the linear range of GTPase reaction. Arachidonic acid (Sigma) and phosphatidic acid (β-arachidonoyl γ-stearoyl) (Avanti Polar Lipids Inc. or Sigma) and phosphatidic acid (dipalmitoyl) (Sigma) were dissolved in chloroform (10 mg/ml). 30 μl of this solution were dried under nitrogen in a glass tube (12 × 75 mM). The thin layer of lipid coating the glass was suspended in 300 μl of 0.1 M Tris−Cl, pH 7.5 and sonicated in a water/ice bath for 30 s by inserting a titanium microtip (Fisher, Model 300 Sonic Dismembranator). Lipids were prepared fresh prior to each assay. Filter binding GTPase assay with yeast RAS2 and IRA2 proteins was performed as described by Tanaka *et al.* (1991).

Immunoprecipitation of Ras by monoclonal antibody Y13-259 was performed as described (Tsai *et al.*, 1989b). Ha-Ras was incubated for 5 min at 30°C with 1 μM [α-^{32}P]GTP (3000 Ci/mmol; NEN) in 50 μl of Tris−Cl, pH 7.5 buffer containing 2 mM dithiothreitol as described above. GTPase reaction was initiated by addition of $MgCl_2$ and GAP 702 or NF1 proteins in 60 μl of reaction buffer (final concentrations: 20 mM Tris−Cl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT and 0.5 mM cold GTP) with or without lipids. After incubation at 30°C for 15 min Ras was immunoprecipitated by monoclonal antibody Y13-259 and protein A−Sepharose beads coated with rabbit antibody to rat immunoglobulin G. Bound nucleotides were released from the immunoprecipitate by boiling for 3 min. Bound nucleotides were resolved on a polyethyleneimine cellulose TLC plate (EM Science) with a 1 M potassium phosphate, pH 3.4 mobile phase, and visualized by autoradiography.

The inhibition of GTPase activity by added lipids was calculated first by determining percentage of bound GTP observed in reactions with both added lipids and GTPase activating proteins and subtracting it from the value observed in reactions with added GTPase activating proteins only. The resulting value was then divided by the percentage of GTP bound to Ras in the absence of all other added materials minus the percentage observed in the presence of added GTPase activating protein only.

Extraction of lipids from the thin layer chromatography plate

One milliliter of chloroform solution of phosphatidic acid (β-arachidonoyl-γ-stearoyl) (5 mg/ml) was, under the nitrogen, reduced in volume to 100 μl and applied to silica gel G60 thin-layer chromatography (TLC) plates (Whatman). The plate was developed with heptane−isopropyl ether-acetic acid (60:40:4). The silica gel bands corresponding to the phosphatidic acid and arachidonic acid (as determined by comparison to the parallel plate stained for lipids) were scraped from the plate with a spatula and extracted with 1 ml of chloroform:methanol (1:1). After centrifugation at 3000 *g* for 5 min, supernatants were transferred to other glass tubes and immediately tested for the ability to inhibit GTPase activity of NF1 and GAP proteins.

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