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Coupling of ras p21 Signalling and GTP Hydrolysis by GTPase Activating Proteins [and Discussion]

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Coupling of ras p21 signalling and GTP hydrolysis by GTPase activating proteins

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SUMMARY

Ras p21 proteins cycle between inactive, GDP-bound forms and active GTP-bound forms. Hydrolysis of bound GTP to GDP is mediated by proteins referred to as GAPs, two forms of which have been described. The first, p120-GAP, contains regions of homologies with tyrosine kinase oncogenes, and interacts with tyrosine phosphoproteins as well as with ras proteins; p120-GAP may therefore connect signalling pathways that involve tyrosine kinase and ras p21 proteins. The second type of GAP is the product of the neurofibromatosis type 1 gene (NF1-GAP). This is a protein of 325 000 Da that is defective in patients with NF1; NF1-GAP is regulated by signalling lipids, and may serve to connect ras p21 with phospholipid second messenger systems. The significance of ras p21 interaction with distinct GAPs is discussed.

1. INTRODUCTION

Ras p21 proteins are members of a large, highly conserved family of GTPases that regulate many aspects of cell growth and behaviour. This family includes rho proteins, which are involved in organisation of the cytoskeleton, rab proteins, involved in vesicular transport, and the ras proteins themselves, which control unknown aspects of proliferation and differentiation. Ras p21 proteins cycle between active, GTP-bound forms, and inactive, GDP-bound forms. Exchange of GDP for GTP is mediated by proteins referred to as guanine nucleotide releasing proteins (GNRPs) or GDP/GTP exchange factors. Very little is currently known about mammalian GNRPs, although the yeast counterpart, the product of the CDC25 gene, has been quite well characterized. More is known about proteins that convert GTP-bound ras p21 to the GDP-bound form. These proteins are referred to as GTPase activating proteins (GAPs) (McCormick 1990). Two distinct types of mammalian GAP have been described: p120-GAP is a protein of 120 000 Da that includes domains with homology to non-receptor tyrosine kinases, the SH2-regions and SH3-regions, and is able to interact with several tyrosine phosphoproteins in addition to ras p21. NF1-GAP is the product of the neurofibromatosis type 1 (NF1) locus, and is predicted to be about 325 000 Da. It appears to be the mammalian homologue of the yeast proteins IRA1 and IRA2 (Xu *et al.*, 1990*a*, *b*; Martin *et al.* 1990; Ballester *et al.* 1990). Both types of GAP appear to be expressed ubiquitously (Wallace *et al.* 1990), and both contribute to ras p21 regulation (Bollag & McCormick 1991).

Biochemical investigation of the interaction between GAP and a number of ras p21 mutants has led to the suggestion that GAPs are necessary for ras function, as well as being essential for their regulation (Hall 1991; Adari *et al.* 1988; Cales *et al.* 1988). For example, point mutants of ras p21 that interact poorly with GAPs are biologically inactive, whereas oncogenic mutants, which are biologically hyperactive, retain GAP binding and, in some cases, bind GAP much more tightly than their wild-type counterparts. GAP binding to ras p21 mutants therefore correlates, in a general sense, with their biological activity. The basis of oncogenic activation by point mutants such as valine-12 or leucine-61 is now understood: GTP bound to these ras p21 proteins is not hydrolysed to GDP by GAP: they therefore escape from down-regulation by GAP but retain GAP binding.

Here we summarize biochemical properties of the ras p21/p120-GAP interaction and the ras p21/NF1-GAP interaction, and present a model for dual effector and regulatory roles of both GAPs in ras function.

2. BINDING OF p120-GAP TO TYROSINE PHOSPHOPROTEINS

It is known that p120 binds to activated PDGF-receptors (Kaplan *et al.* 1990; Kazlauskas *et al.* 1990) and with cellular phosphotyrosine proteins referred to as p190 and p62 (Ellis *et al.* 1990). The region of GAP responsible for these interactions has been determined by testing the ability of a series of GAP mutants to associate with these substrates (figure 1).

(*a*) *PDGF-receptor*

Murine PDGF-receptor was produced in insect cells. Membranes from infected cells were incubated with ATP to activate these receptors to facilitate

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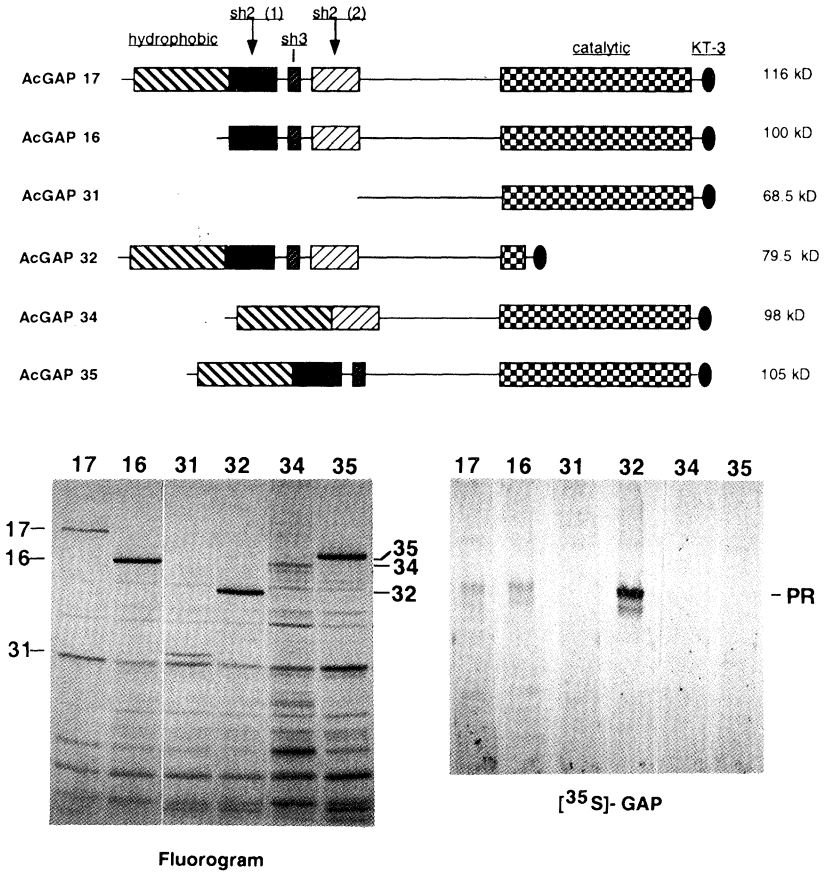

Figure 1. Structure of GAP mutants and their association with activated PDGF-receptor. GAP mutants containing a C-terminal epitope Tag (Martin *et al.* 1990) were expressed in insect Sf 9 cells, and labelled metabolically with [^{35}S]methionine. Total extracts from infected cells were resolved by SDS-PAGE, and recombinant GAP mutants visualized by autoradiography (left panel). Labelled mutants were incubated with activated PDGF-receptors (Fantl *et al.* 1992), and washed filters were analysed for bound GAP by using autoradiography (right panel).

autophosphorylation and hence receptor activation. Activated receptors were then resolved on SDS-PAGE, and transferred to nitrocellulose filters. These filters were incubated with [^{35}S]GAP mutants, and the ability of these mutants to bind directly to the receptors judged by autoradiography. Figure 1 shows the results of such an experiment. Full-length p120-GAP and p100-GAP (an alternative-splice variant expressed exclusively in placental tissue) bound efficiently to activated receptors. In a separate experiment (not shown), binding was shown to be dependent on autophosphorylation of the receptor. GAP mutants that contain only one SH2 region, or no SH2 regions at all, fail to bind to the receptor, whereas a GAP mutant (GAP32) that lacks the site of ras p21 interaction (the C-terminal 334 amino acids) binds efficiently. Indeed, this mutant appears to bind more efficiently than does wild type GAP. We therefore conclude that the SH2/SH3 region is required for binding, whereas interaction with ras p21 is not.

(*b*) *Association of GAP with p62 and p190*

The same panel of mutants that were tested for PDGF-receptor association was tested for binding to the cellular tyrosine phosphoproteins p62 and p190. Once again, the SH2/SH3 regions were necessary for binding, and the ras p21 binding domain was dispensible for this interaction. These results also showed that GAP itself does not appear to require tyrosine phosphorylation to bind to PDGF-receptor, p62 or p190, as we cannot detect significant levels of phosphotyrosine in baculovirus-produced GAP. Furthermore, binding of GAP to these tyrosine phosphoproteins is direct, that is, it does not require any other cellular components.

Table 1. *Summary of effect of GAP mutants on K^+-channel coupling to muscarinic receptors*

GAP mutant[a]	inhibition of coupling[b]	ras p21 dependence[c]
GAP 17	+ + + +	yes
GAP 16	+ + + +	yes
GAP 31	—	—
GAP 32	+ + +	no
GAP 34	+	no data
GAP 35	+ +	yes

[a] See figure 1 for structures of these mutants.
[b] For details, see Yatani *et al.* (1990) and Martin *et al.* (1992).
[c] Ras p21 dependence was judged by the ability of anti-ras antibody Y13-259 to prevent GAP-mediated inhibition (Yatani *et al.* 1990).

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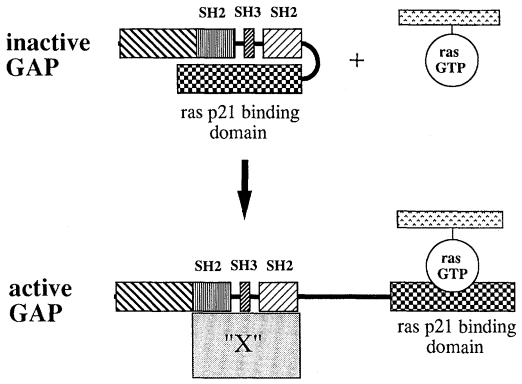

Figure 2. Model for the regulation of GAP binding to tyrosine phosphoproteins by ras p21.

(*c*) *Inhibition of G-protein coupling*

Full-length GAP inhibits coupling of M2 muscarinic receptor to the heterotrimeric G-protein G_k in patch clamped membranes from guinea-pig atrial cells (Yatani *et al.* 1990). Inhibition appears to be ras p21 dependent, as prior incubation of the membrane with anti-ras antibodies prevents the GAP effect. The ability of GAP mutants to inhibit coupling was tested in this system, and the results (Martin *et al.* 1992) are summarized in table 1. Once again, the SH2 regions were necessary for the effect, a fragment of GAP consisting of the SH2/SH3 regions giving the strongest effect. GAP mutants containing the ras p21-binding domain were sensitive to anti-ras p21 antibodies, suggesting that they require ras p21 interactions for their effects. However, GAP mutants lacking this domain were insensitive to these antibodies, and therefore functioned in a ras-independent manner. We conclude that the ras p21 binding domain normally inhibits interaction of GAP with its target in these membranes, and that ras p21 functions by relieving this inhibition. Removal of the domain results in loss of this requirement for ras interaction, as depicted in the model in figure 2. We speculate that the increased binding of GAP32 (the form that lacks the ras p21 domain) to PDGF-receptor may also reflect relief from a constraint imposed by the ras p21 binding domain on the SH2 region, and that ras p21 may increase binding of GAP to the receptor. This is currently being tested.

3. PROPERTIES OF NF1-GAP

(*a*) *Effects of NF1-GAP on wild-type ras p21*

At nanomolar concentrations of ras p21, the catalytic fragment of NF1-GAP is about as active as p120-GAP in converting ras p21-bound GTP to GDP. At micromolar concentrations of ras p21, p120-GAP is at least 30-fold more efficient than NF1-GAP. This is because NF1-GAP has a lower K_m for ras p21.GTP than does p120-GAP, but both have similar catalytic constants: the V_{max} for NF1-GAP is therefore achieved at ras p21 concentrations 30-fold lower than for p120-GAP, and this V_{max} is 30-fold lower than for p120-GAP. The affinity of NF1-GAP and p120-GAP for ras p21 was measured directly, and the results are summarized in table 2 (Bollag & McCormick 1991). The apparent K_d of NF1-GAP for H-ras p21 is 35 nM, compared with 5 μM for p120-GAP. Of significant interest is the observation that NF1-GAP has a different binding constant for H-ras and N-ras p21 proteins. This suggests that the binding site for NF1-GAP on ras p21 includes residues at which H-ras and N-ras differ. Most of these differences are the carboxy-terminal half of the ras p21 protein. However, p120-GAP binds identically to H-ras and N-ras, confirming previous suggestions that this interaction is limited to highly conserved aminoterminal regions, including the effector binding site.

Table 2. *Binding constants of ras mutants p120-GAP and NF1-GAP*[a]

ras p21 protein	p120-GAP binding/nM	NF1-GAP binding/nM
H-ras (wild-type)	5000	35
H-ras D38A	> 10 000	5000
H-ras Q61L	500	2
H-ras Q61H	2000	25
N-ras (wild-type)	5000	160
N-ras G12D	5000	160

[a] Taken from Bollag & McCormick (1991).

(*b*) *Effects on ras p21 mutants*

Oncogenic mutants of ras p21 are resistant to the GTPase-activating effects of both p120-GAP and NF1-GAP (see table 2, taken from Bollag & McCormick 1991). However, these mutants still bind to both GAPs: the Asp-12 mutant of N-ras p21 binds with a similar affinity to wild-type Gly-12 N-ras p21. However, position 61 mutants have much higher affinities than their wild type counterparts. Leu-61 H-ras, in particular, binds to NF1-GAP with a K_d of 2 nM, compared with 100 nM for wild-type H-ras. In all cases, binding to NF1-GAP was much tighter than to p120-GAP. Again using Leu-61 as an example, this ras p21 mutant binds NF1-GAP 250 times more tightly than p120-GAP.

The Ala-38 mutant of v-H-ras is unable to transform cells, even though it binds GTP normally and localizes in the plasma membrane. Ala-38 therefore constitutes part of the 'effector' binding site, a region that is essential for interaction between ras p21.GTP and target molecules involved in signal transmission. This point mutation reduces the affinity of ras p21 for NF1-GAP by 150-fold (K_d of 5 μM instead of 35 nM for wild-type H-ras p21). This suggests either that NF1-GAP is necessary for biological activity of ras proteins, or that other proteins which are necessary bind to ras p21 at a site similar to the binding site for NF1-GAP.

(*c*) *Effects of lipids on NF1-GAP activity*

Lipids inhibit the ability of NF1-GAP to stimulate ras p21 GTPase at concentrations of 10–100 μM. Of these,

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PIP2 is the most potent that we ahve tested so far (Bollag & McCormick 1991). Inhibition was shown to be non-competitive, suggesting that ras p21 and lipid bind to NF1-GAP at different sites. Indeed, we have shown that ras p21 binding to NF1-GAP is unaffected by concentrations of lipid that totally block GTPase activity. Wild-type p21, in its GTP-bound form, has the potential of forming a stable complex with NF1-GAP in the presence of lipids. It is therefore possible that during activation of cells by mitogens, lipids are produced that protect ras p21.GTP from hydrolysis by GAPs by allowing stable association of NF1-GAP.

4. DISCUSSION

Figure 3 shows a model in which signal output from ras p21 is mediated by p120-GAP and NF1-GAP, and in each case the signal is coupled to GTP hydrolysis. This model will be discussed in the context of normal cells, ras-transformed cells and NF1 cells.

(*a*) *Regulation of ras p21 by GAPs in normal cells*

GNRPs are responsible for conversion of inactive, GDP-bound ras p21 to the active, GTP-bound form. This activation event is expected to be regulated, as GDP/GTP exchange is rate limiting in the cycle shown in figure 3 (in normal cells, most of the ras p21 is in the GDP state). Ras p21.GTP generated by the action of GNRPs is brought back to the inactive state by one of two parallel paths, one using p120-GAP, the other using NF1-GAP. Support for this comes from the discovery that most cells express both types of GAP activity, at similar levels of activity (Bollag & McCormick 1991). At low concentrations of ras p21, both pathways are likely to make similar contributions to ras p21 GTP hydrolysis, as both p120-GAP and NF1-GAP have similar enzymatic activities under these conditions. Activation of T-cells by antibodies against T-cell receptor or TPA (Downward *et al.* 1990), or of fibroblasts by insulin (Burgering *et al.* 1991), results in a rapid increase in the ratio of ras p21.GTP: ras p21.GDP. To increase the ratio of GTP: GDP bound to rasp 21 from 10% to 90% (roughly the change reported for these cells), a drop in GAP activity of 100-fold is necessary, as discussed elsewhere (McCormick *et al.* 1988), assuming a constant GNRP activity. Because T-cells and fibroblasts contain both p120-GAP and NF1-GAP activities, we assume that both types of GAP are inhibited by cell activation, because complete inactivation of either GAP could not cause such a drop in total GAP activity. The mechanism of GAP inactivation in these cells is currently under investigation.

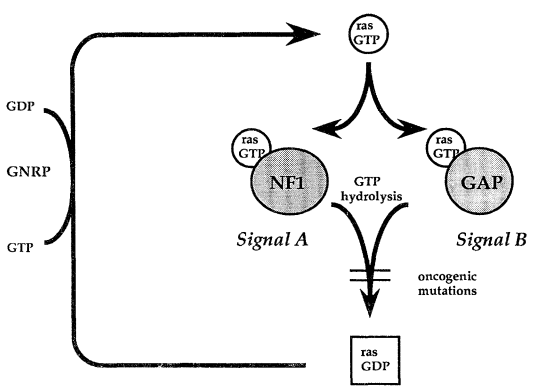

Figure 3. Model for control and signalling from ras p21 involving parallel p120-GAP and NF1-GAP pathways.

As proposed in figure 3, interaction of ras p21.GTP with p120-GAP and NF1-GAP generates different cellular signal outputs. As discussed above, p120-GAP may be involved in coupling ras p21 to signalling pathways involving tyrosine phosphoproteins. We speculate that interaction of p120-GAP with these targets is ras p21 dependent, and that ras p21 binding to p120-GAP causes a conformational change that allows SH2/SH3 regions of p120-GAP to bind to these targets. We do not know the functional significance of p120-GAP binding to cellular phosphoproteins such as PDGF-receptor, but suggest, from a number of indirect observations, that p120-GAP is necessary for effective signalling from this receptor.

If indeed NF1-GAP is another ras effector, as suggested by analysis of binding properties of mutant ras proteins, we assume that signals generated from interaction of ras p21 with NF1-GAP are distinct from those emitted from p120-GAP. This is simply because these GAPs share no sequence similarities outside the ras-binding region. NF1-GAP has no SH2 sites, for example.

Whether GAPs are effectors or not, it is clear from the figure that the parallel pathways of ras p21 down-regulation are interdependent, as they compete for the same substrate (ras p21.GTP). Increased activity of one pathway is expected to decrease the activity of the other by reducing the level of p21 in the GTP state. This may be an important mechanism for exerting dual control over two distinct signalling pathways in the cell.

(*b*) *Interactions between ras p21.GTP and GAPs in ras-transformed cells*

Oncogenic ras p21 proteins are insensitive to p120-GAP and NF1-GAP, and therefore remain in their active, GTP states constitutively. The proportion of oncogenic ras p21 bound to p120-GAP relative to NF1-GAP will differ between specific ras p21 mutants: for leu-61, for example, most of the ras p21 may be bound to NF1, because the binding constant for this interaction is 2 nM, about 100-fold tighter than for p120-GAP. This presents a possible explanation for the failure of p120-GAP to bind PDGF-receptor in cells transformed with leu-61 ras p21: according to the model discussed above, p120-GAP may require ras p21 for efficient binding to PDGF-receptor. If most of the leu-61 ras p21 protein is tightly associated with NF1-GAP, the ability of p120-GAP to interact with its targets may be impaired.

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(*c*) *ras p21 and GAPs in NF1 cells*

Neurofibromatosis type 1 is a disease characterized by abnormal proliferation of cells derived from the neural crest, such as Schwann cells and melanocytes. In cells of neural crest origin, activated ras p21 causes growth arrest or differentiation. We would therefore assume that activated forms of ras p21 would not occur in tumours of neural crest origins. Consistent with this assumption, we have been unable to detect activated ras oncogenes in tumours of neural crest origin from NF1 patients (Markert *et al.*, submitted). It therefore appears that in cells affected by NF1 the signal emitted from ras p21 is coupled to a negative growth pathway, so that loss of ras effector function may contribute to uncontrolled growth. We propose that the loss of one functional NF1 allele causes a reduced signal output from ras p21, or possibly interference with normal NF1 function, and that this is the basis of the disease.

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Discussion

K. C. Holmes (*Max-Planck-Institüt für medizinische Forschung, Heidelberg, F.R.G.*). Is the SH2 region like a protein kinase in sequence?

F. McCormick. No. It recognizes the phosphorylated form of tyrosine in a specific sequence. For example, GAP binds to these residues in the PGF receptor, but proteins like src have SH2 sites within themselves and it is thought they fold over on themselves.

K. C. Holmes. It is just like one of the domains of src, which is not the kinase.

F. McCormick. Exactly, SH1 is the kinase region.

R. H. Michell (*Department of Biochemistry, University of Birmingham, U.K.*). Given the differing affinities of NF1 and p120-GAP for ras, and the likelihood that they mediate different signalling pathways in cells, what are the relative intracellular concentrations of these proteins in cells, and how do these concentrations vary between cells?

F. McCormick. The answer is not yet known. The trivial problem is that there are no good antibodies yet

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to NF1. The other problem is, at least for the p120-GAP, a large amount is associated with this protein p190 which does not interact with p21ras, so it is difficult to assess how much is available to interact with p21ras. By using a biochemical trick to distinguish between the two activities, the relative activities have been measured and they are roughly equal. In terms of total mass of proteins that are able to interact, we do not have any idea.

R. H. Michell. The experiments with Buzz Brown clearly indicate ras-GAP modulation of receptor-G-protein coupling to K^+ channels in atrial membranes. Given the history of controversial claims of effects of p21ras on receptor-G-protein coupling (e.g. enhancement or blockade of receptor-phosphoinositidase C coupling), is there any evidence for ras-GAP interactions with other receptor-activated and G-protein-coupled signalling pathways?

F. McCormick. Attempts to find similar systems *in vitro* have failed. We have some peripheral data, in the sense that activation of thrombin receptors in platelets causes phosphorylation of GAP on tyrosine and association of GAP with tyrosine kinases, and thus with p21ras. Thus there is a functional connection between the thrombin receptor and GAP in the whole platelet and also in fibroblasts. It was found that activation of membrane-spanning receptors causes phosphorylation of many proteins on tyrosine. Tyrosine kinases need p21ras for their function, so we have a hint of a connection between G-proteins and tyrosine kinases, but we have not been able to make the connection in any other system except this one. There is a phosphotyrosine-containing protein now in these atrial cell membranes that GAP binds, and that may be the candidate for the protein that somehow regulates this interaction. We feel there is a connection but have not really made it except in this one system which is not understood enough yet. There is also a controversy about whether the $\beta\gamma$-subunits are acting on the channel or the α-subunit. It was suggested that $\beta\gamma$-subunits open the channels because the subunits were dissolved in CHAPS detergent. CHAPS liberates phospholipase A2, and that effect may be sufficient. GAP blocks the CHAPS effect in the system.

A. J. Crompton. Does this abnormal protein that Dr McCormick found offer any prospects for treatment for neurofibromatosis?

F. McCormick. One of the goals is to understand the basis of neurofibromatosis, and thus find drugs that would be useful in inhibiting the disease. We are trying to determine whether or not the effects of neurofibromatosis is a defective gene, a result of the dominant interference with the normal gene product, or whether it is just loss of NF1 function. If it is a dominant interference effect then we might imagine drugs that could prevent that happening and would prevent the manifestation of the disease. There are many other ways of attacking the disease. One possibility that has been suggested is that up-regulation of p21ras causes autocrine production of glial cell growth factors, and that inhibitors of glial cell growth factors might prevent proliferation of Schwann cells, for example. Thus the insight that ras and NF1 are involved in the disease is just the first step, and eventually it may have some potential for therapeutics. We certainly hope so.

A. J. Crompton. So Dr McCormick is thinking of small molecules rather than any protein replacement?

F. McCormick. Yes. Unless one can imagine inhibitors of autocrine growth factors, and then inhibitors could be antibodies against the growth factor, or competing proteins, for example.

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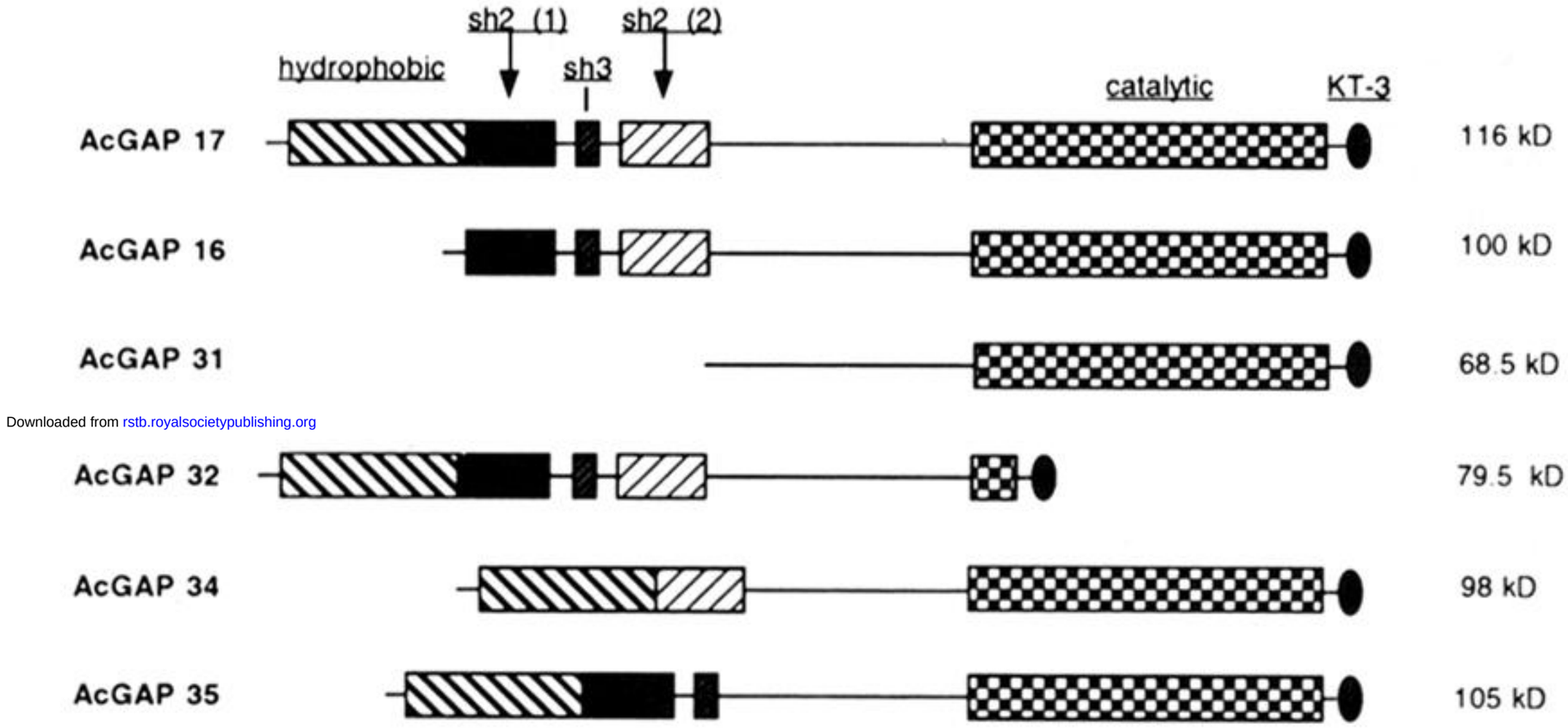

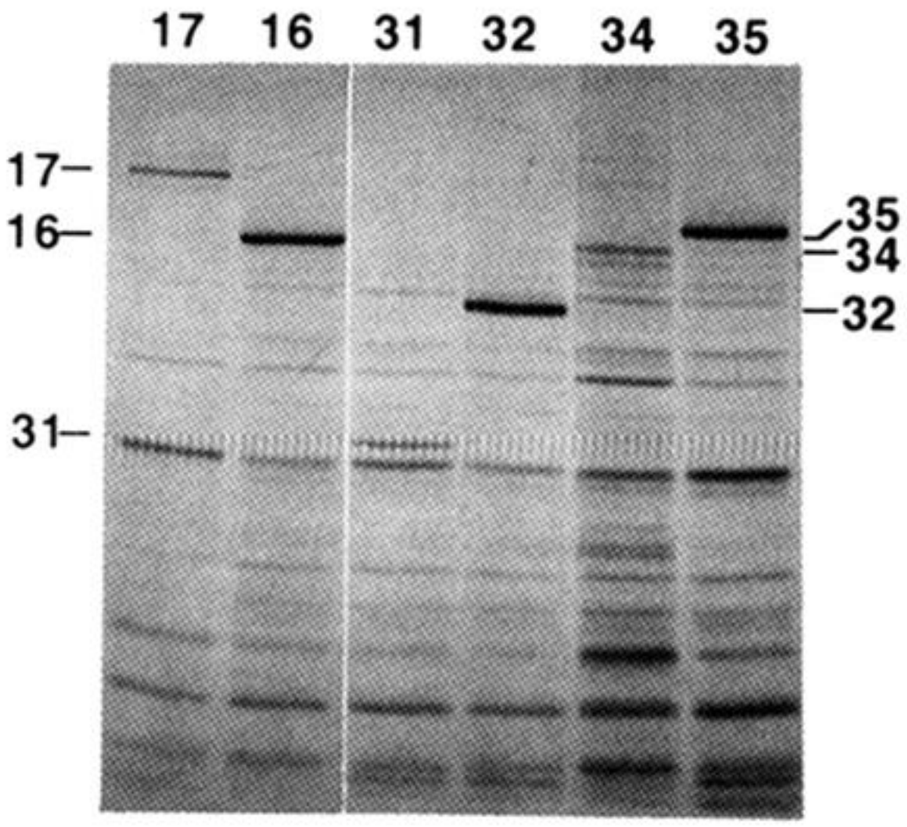

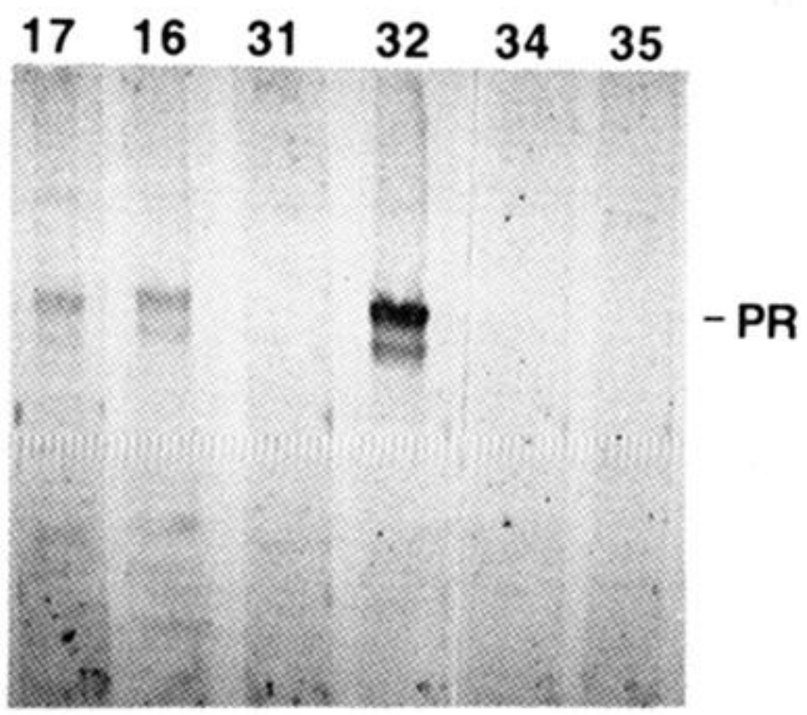

Figure 1. Structure of GAP mutants and their association with activated PDGF-receptor. GAP mutants containing C-terminal epitope Tag (Martin *et al.* 1990) were expressed in insect Sf 9 cells, and labelled metabolically with [^{35}S]methionine. Total extracts from infected cells were resolved by SDS-PAGE, and recombinant GAP mutants visualized by autoradiography (left panel). Labelled mutants were incubated with activated PDGF-receptors (Fantl *et al.* 1992), and washed filters were analysed for bound GAP by using autoradiography (right panel).