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The Kinetic Mechanism of the GAP-Activated GTPase of p21ras [and Discussion]

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Phil. Trans. R. Soc. Lond. B 1992 **336**, 49-54
doi: 10.1098/rstb.1992.0043

The kinetic mechanism of the GAP-activated GTPase of p21ras

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SUMMARY

Guanine nucleotides modified by acetylation of the ribose moiety with the small fluorophore *N*-methylanthranilic acid(mant) have been shown to bind to p21ras with similar equilibrium and kinetic rate constants as the parent nucleotides. Hydrolysis of p21.mantGTP to p21.mantGDP results in a 10% decrease in fluorescence intensity occurring at the same rate as the cleavage step. A similar process occurs with the non-hydrolysable analogue mantGMP.PNP, and this has led to the proposal that a conformational change of p21.mantGTP precedes and controls the rate of the cleavage step. The fluorescence change with p21.mantGMP.PNP is accelerated in the presence of the C-terminal catalytic domain of GAP, which is consistent with this mechanism. The same conformational change does not occur with oncogenic mutants of p21ras, Asp-12 and Val-12, but does occur with the weakly oncogenic Pro-12 mutant. Stopped flow measurements of the interaction of GAP with p21.mantGTP show an exponential decrease in fluorescence, the rate of which does not vary linearly with GAP concentration. These data imply a rapidly reversible formation of the p21.mantGTP complex with GAP followed by the isomerization of this complex. This is at least 10^5-fold faster than the same process in the absence of GAP.

1. INTRODUCTION

The products of the N-ras, H-ras, and K-ras genes, termed p21 proteins, are low molecular mass proteins (21 kDa) which bind GTP and GDP and have a low intrinsic GTPase activity. The three proteins are highly homologous, mainly differing in the C-terminal 20 amino acids where post-translational modification occurs, enabling them to become associated with a membrane. Single point mutations at certain sites in the proteins lead to oncogenic properties. As with other guanine nucleotide-binding proteins, they exist in a biologically active state when GTP is bound, and hydrolysis to the GDP bound form results in the formation of an inactive state. Dissociation of GDP from the protein is followed by the reformation of the active GTP-bound species. The relative concentrations of p21.GTP and p21.GDP in the cell are therefore governed by the relative rate constants of the hydrolysis of p21.GTP and of the dissociation of GDP from p21.GDP. With purified recombinant proteins, these are both very slow processes with rate constants of about $3 \times 10^{-4}\,s^{-1}$ at 37°C, giving half-times of about 40 min for both processes. However, other proteins have been identified which accelerate these processes and so can control the ratio of p21.GTP and p21.GDP in the cell. Two GTPase activating proteins (GAPs) have been isolated, p120-GAP and NF1-GAP, both of which accelerate the rate of conversion of p21.GTP to p21.GDP. The factors which accelerate the rate of release of GDP from p21.GDP, and hence the rate of conversion of p21.GDP to p21.GTP, are much less well defined, although a factor for the related smgp21 has been characterized (Takai 1992). The properties of p21ras, GAP, and nucleotide exchange factors summarized above have been reviewed by, amongst others, Barbacid (1987), Bourne *et al.* (1991), Grand & Owen (1991), Goody *et al.* (this symposium) and McCormick (this symposium).

To understand how the ratio of p21.GTP to p21.GDP is controlled in the cell, and how single point mutations cause this to be disrupted, we have investigated the kinetic mechanism of the p21ras GTPase activity. We have defined the important intermediates in the GTPase cycle, measured the rate constants of their interconversion, and identified a structural change occurring between p21.GTP and p21.GDP which may be related to its biological function. The main approach used is to design experiments in which the process of interest only occurs once on any given p21 molecule (i.e. single turnover conditions) combined with the use of well-defined complexes of p21 with guanine nucleotide. These methods have proven successful with other GTPase and ATPase mechanisms, such as the myosin ATPase (Trentham *et al.* 1974), the elongation factor Tu GTPase (Eccleston *et al.* 1985) and the mechanism of release of GDP from elongation factor Tu catalysed by elongation factor Ts (Eccleston 1984). They are complementary to the X-ray diffraction studies of complexes of p21 with guanine nucleotides reported by Goody *et al.* (this symposium). The advantage of

Phil. Trans. R. Soc. Lond. B (1992) **336,** 49–54
Printed in Great Britain

the methods described here lie in the fact that measurements can be made on a millisecond time scale in solution where crystal packing restraints are not present.

Initially we studied the intrinsic GTPase mechanism of p21 in the absence of any other factors, and proposed a basic mechanism involving GTP binding, the cleavage step, and then the release of P_i and GDP. From studies with a fluorescent analogue of GTP we then proposed that the cleavage step was controlled by a preceding isomerization of the p21.GTP complex. We are now investigating how single point mutations and the presence of GAP affect this mechanism.

2. BASIC MECHANISM OF GTP HYDROLYSIS BY $p21^{N\text{-}ras}$

The simplest mechanism for the hydrolysis of GTP by $p21^{N\text{-}ras}$ is given in scheme 1, where p21 is represented by R.

$$R + GTP \overset{1}{\rightleftharpoons} R.GTP \overset{2}{\rightleftharpoons} R.GDP.P_i \overset{3}{\rightleftharpoons} R.GDP + P_i \overset{4}{\rightleftharpoons} R + GDP$$

Scheme 1.

For the ith step the equilibrium constant is given by K_i and the forward and backward reaction rate constants are given by k_i and k_{-i}, respectively.

We initially measured most of the rate constants in this scheme for wild type N-ras and two oncogenic mutants by using tritiated nucleotides. Reactions were analysed either by high performance liquid chromatography (HPLC) to separate [^{3}H]GTP from [^{3}H]GDP or by filter binding assays to separate protein-bound nucleotide from free nucleotide (Neal *et al.* 1988). k_1 and k_{-4} were measured by mixing nucleotide-free p21 under pseudo-first-order conditions with [^{3}H]GTP or [^{3}H]GDP (i.e. [nucleotide] $\gg$ [p21]) and following the binding of nucleotide with time. Because this is a fast reaction and needed manual sampling, it could only be measured over a limited (nM) concentration range at 0°C, but the rate constants were shown to be second order over the range 0–25 nM guanine nucleotide. At 10°C, only the pseudo-first-order rate constant at a single concentration was measurable. All of the remaining experiments were done at 37°C. The values of k_{-1} and k_2 were measured by a cold chase experiment where p21.[^{3}H]GTP was added to an excess of GTP. The [^{3}H]GTP could either dissociate or go through the cleavage step. The analysis of the data is as described by Bagshaw & Trentham (1973), but essentially the observed rate constant of hydrolysis of [^{3}H]GTP and the proportion of [^{3}H]GTP and [^{3}H]GDP at the end of the reaction allows the calculation of these two rate constants. $(k_2 + k_{-2})$ could be measured independently by preparation of the p21.[^{3}H]GTP complex and following the rate of formation of [^{3}H]GDP, and the end point of formation of [^{3}H]GDP, allowed an upper limit of k_{-2} to be obtained. k_4 was obtained by addition of an excess of GDP to a complex of p21.[^{3}H]GDP, and following the dissociation of [^{3}H]GDP. Information on k_3 and k_{-3} was obtained by the methods described by Webb (this symposium).

The principal results from this work were that the steps contributing most to the overall catalytic rate of GTP hydrolysis were the cleavage step (k_2) and the GDP dissociation step (k_4), the other forward steps being fast and the reverse rate constants being slow. Although the oncogenic mutant proteins Asp-12 and Val-12 had different values of k_2 and k_4, they did not make a significant difference to the ratio of p21.GTP to p21.GDP in steady-state conditions with an excess of GTP. These values of k_1 and k_{-1} and of k_4 and k_{-4} also allowed a determination of the equilibrium constants K_1 and K_4 which gave values showing much tighter binding than previously measured. As discussed by Goody *et al.* (1991), this kinetic determination of the association equilibrium constants of guanine nucleotides to p21 is superior to that of equilibrium methods. The latter have the disadvantage that the nucleotide-free protein is unstable with respect to the time required for equilibrium to be obtained. This problem is compounded by many workers measuring the equilibrium between p21.GDP (rather than nucleotide-free p21) with p21.[^{3}H]GDP, which completely invalidates their analysis. However, the kinetic method of measuring the equilibrium constants should also be treated with caution because it involves extrapolating second-order rate constants (and one of these from a single concentration) from between 0°C and 10°C to 37°C, and assuming a linear Arrhenius plot.

3. DO CONFORMATIONAL CHANGES OCCUR DURING THE MECHANISM?

The mechanism in scheme 1 only includes steps involving binding, chemical cleavage and product release. However, we know that structural changes probably occur during the transition between p21.GTP and p21.GDP based on their different biological activities. Also, isomerizations are important processes in the kinetic mechanism of other nucleoside triphosphatases such as the actomyosin ATPase (Geeves, this symposium). Structural changes in p21ras have also been demonstrated by X-ray crystallography data (Schlichting *et al.* 1990). To explore the p21.GTPase mechanism further, we used fluorescent analogues of guanine nucleotides in which the *N*-methylanthraniloyl group was introduced onto the 2′ and 3′ positions of the ribose moiety. These nucleotides exist as an equilibrium mixture of the 2′ and 3′ isomers when free in solution, but more than 97% of the nucleotide bound to p21 in all the complexes studied was the 3′ isomer (Eccleston *et al.* 1991).

The equilibrium and kinetic constants of the mant-nucleotides in their interaction with p21 are all within a factor of two of those of the parent nucleotides, showing them to be good analogues for studying the system. Furthermore, mantGTP and mantGDP showed enhancements in fluorescence of 3.2- and 2.8-fold respectively on binding to p21. This enabled the

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rates of association of nucleotides to p21 (John *et al.* 1990), dissociation of nucleotides and the cleavage step to be measured by fluorescence measurements (Neal *et al.* 1990). The association rate constants measured in this way gave values inconsistent with those using the filter binding method with [^{3}H]GTP and [^{3}H]GDP, and this led to the proposal of more complex nucleotide binding schemes than a simple second-order process. However, the rate constant of the fluorescence change occurring on the cleavage step was identical to that measured chemically, and that of the fluorescence change occurring on diphosphate dissociation was identical to that measured by gel filtration.

The fluorescence change occurring at the same rate as the cleavage step on p21.mantGTP hydrolysis can be interpreted in terms of three possible mechanisms: (i) a conformational change occurs during the cleavage step, (ii) a conformational change occurs during the phosphate release step which is fast compared to the cleavage step; and (iii) a conformational change occurs before, and limits the rate of, the cleavage step. We proposed the last mechanism is applicable (scheme 2) based on experiments using a complex of p21 with mantGMP.PNP (Neal *et al.* 1990). This analogue is not hydrolysed by p21 over the time course of our experiments, but on incubating a complex of p21.mantGMP.PNP at 37°C we observed an exponential decrease in fluorescence occurring with a rate constant similar to that with mantGTP.

$$\text{R.mantGTP} \overset{2a}{\rightleftharpoons} \text{R.mantGTP*} \overset{2b}{\rightleftharpoons} \text{R.mantGDP.P}_i$$

Scheme 2.

4. DOES THE SAME CONFORMATIONAL CHANGE OCCUR WITH POSITION 12 MUTANTS?

The replacement of Gly-12 with any amino acid results in an oncogenic protein, although if the amino acid is proline it is only weakly oncogenic. We measured the cleavage rates of the wild type Gly-12 protein and of the Asp-12, Val-12 and Pro-12 mutant proteins and found them to be similar, Asp-12 and Val-12 being slightly slower than wild type and Pro-12 being slightly faster. Measuring fluorescence during the mantGTP hydrolysis process we observed a 10% decrease in fluorescence intensity with the Gly-12 and Pro-12 proteins; with Asp 12- and Val-12 no fluorescence change associated with the cleavage reaction occurs. This suggests a correlation between the lack of transforming ability and the presence of the fluorescence change.

5. EFFECT OF GAP ON THE p21.GTPase MECHANISM

The mechanism proposed in scheme 2 for the hydrolysis of GTP by p21 is that the cleavage step is controlled by a preceding isomerization of the p21.GTP complex which gives rise to the fluorescence change on incubating p21 with either mantGTP (when hydrolysis occurs) or with mantGMP.PNP (when hydrolysis does not occur). As GAP accelerates the rate of cleavage of GTP bound to p21, this mechanism predicts that GAP would accelerate the rate of the fluorescence change occurring with either mantGTP or mantGMP.PNP.

We initially did experiments to show that mantGTP closely followed the properties of GTP itself in the GAP catalysed hydrolysis. We showed that with wild type p21N-ras, in the absence of GAP, the rate of mantGTP hydrolysis was 67% that of GTP. However, in the presence of between 0 and 3 μM GAP (and at 15 μM p21.GTP or p21.mantGTP), there was a linear dependence of both cleavage rates, although mantGTP was hydrolysed threefold faster than GTP. Following the fluorescence of the solution of p21.mantGTP with time showed an exponential 10% decrease, which occurred at the same rate as the cleavage reaction over the range of 0 to 3 μM GAP.

The crucial test of scheme 2 was to investigate the effect of GAP on the fluorescence process occurring with mantGMP.PNP, because in this situation no hydrolysis occurs. Although the amplitude of this process is now only a 5% decrease, we showed that it was accelerated in a linear dependent manner at GAP concentrations between 0 and 3 μM (15 μM p21.mantGMP.PNP), although at a threefold lower extent than that of mantGTP. This provides good evidence that the cleavage step is controlled by a preceding isomerization of the p21.mantGTP complex, and it is this process that is accelerated by GAP.

6. ELEMENTARY RATE CONSTANTS OF THE GAP-ACTIVATED p21.GTPase MECHANISM

Although the above experiments have shown that it is probable that GAP accelerates the isomerization of the p21.mantGTP complex, they do not give any information about the elementary rate constants of the GAP-activated hydrolysis which are needed to define the important intermediates in this process. We therefore did experiments in which GAP was in a large excess over p21.mantGTP so that only a single turnover of p21.mantGTP occurred. This resulted in a fast reaction which required the use of a stopped flow fluorimeter, and even though this was designed and built for low volumes of solution it still required relatively large amounts of GAP. The ras GTPase-activating catalytic domain of GAP, which represents its C-terminal 340 amino acids, has similar activity to that of full length GAP (Marshall *et al.* 1989; Skinner *et al.* 1991). This protein was expressed in *E. coli* and purified by using a one-step immunoaffinity column procedure, as it has been engineered to contain a three amino acid epitope to a monoclonal antibody (Skinner *et al.* 1991). Thus sufficiently large amounts of the domain were produced in a highly soluble form.

On mixing 1 μM p21.mantGTP with either 6 μM or 60 μM GAP at 23°C, a decrease in fluorescence occurred (figure 1) which could be fitted to a single exponential. The amplitude of the signal increased with [GAP]. Plotting the observed rate constant

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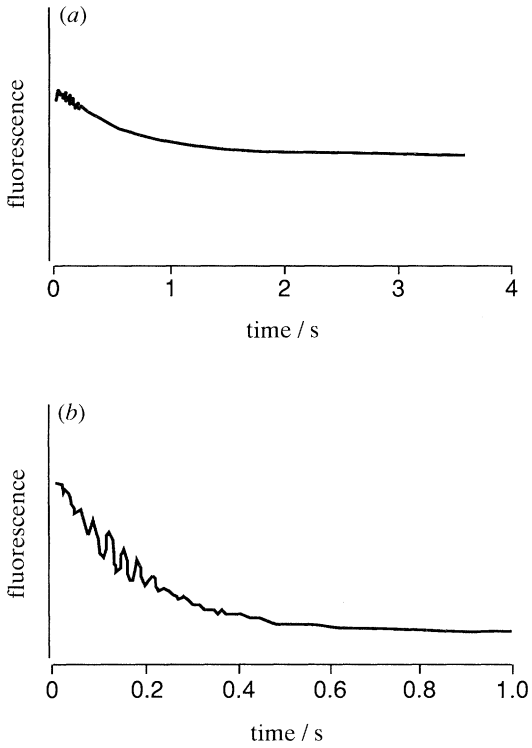

Figure 1. Stopped flow spectrofluorometric record of the reaction of p21.mantGTP with GAP at 23°C. One syringe contained 1 μM p21.mantGTP, and the other syringe contained (*a*) 6 μM GAP and (*b*) 60 μM GAP (final concentrations after mixing). Both syringes also contained 20 mM trisHCl, pH 7.5, 1 mM $MgCl_2$ and 1 mM dithiothreitol. Excitation was from light from a 100 W mercury arc lamp passed through a 366 nm interference filter. Emission was monitored through a Wratten 47B bandpass filter.

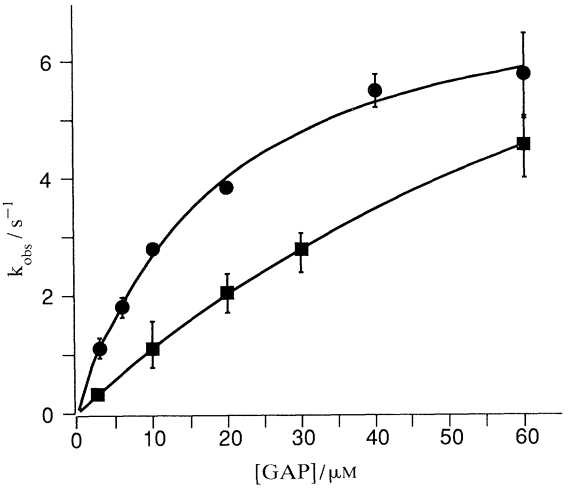

Figure 2. Dependence of the rate constant of GAP binding to p21.mantGTP on GAP concentration. Data as in figure 1 were fitted to a single exponential and the observed rate constant plotted against [GAP]. Solution conditions as in figure 1 (circles); as at figure 1 but also containing 50 mM NaCl (squares).

against GAP concentration showed a hyperbolic dependence at low salt conditions (figure 2). Although such behaviour can result from several mechanisms (as discussed by Bagshaw & Trentham (1973)), the simplest mechanism to explain the results is that an initial complex is rapidly formed between p21.mantGTP and GAP with an increase in fluorescence, and this is followed by the isomerization process.

$$\text{p21.mantGTP} + \text{GAP} \underset{}{\overset{K_1'}{\rightleftharpoons}} \text{p21.mantGTP.GAP} \overset{k_{2a}'}{\rightarrow} \text{p21.mantGTP*.GAP}$$

Scheme 3.

Here $K_1' = 20$ μM (from the [GAP] at half-maximum k_{obs}) and $k_{2a}' = 8\ s^{-1}$ (from the limiting rate constant). By analogy with the mechanisms involving the activation of myosin by actin (see, for example, Geeves, this symposium), we can write a scheme in which GAP can interact with all of the intermediates of the p21.GTPase mechanism.

$$\begin{array}{ccccccc} \text{R.GTP} & \rightleftharpoons & \text{R*.GTP} & \rightleftharpoons & \text{R.GDP.P}_i & \overset{P_i}{\rightleftharpoons} & \text{R.GDP} \\ \downarrow\uparrow K_1' & & \downarrow\uparrow k_{2a}' & & \downarrow\uparrow & & \downarrow\uparrow \\ \text{GAP.R.GTP} & \rightleftharpoons & \text{GAP.R*.GTP} & \rightleftharpoons & \text{GAP.R.GDP.P}_i & \overset{P_i}{\rightleftharpoons} & \text{GAP.R.GDP} \end{array}$$

Scheme 4.

We can assign K_1' to the initial binding process and k_{2a}' to the isomerization in the presence of GAP. Although ionic strength conditions vary between experiments in the presence and absence of GAP reported here, it can be seen that the isomerization is accelerated by a factor of 10^5 in the presence of GAP.

In the presence of 50 mM NaCl, this concentration dependence is markedly changed. GAP concentrations high enough to cause saturation of the observed rate constant were not reached, and the data cannot be analysed with the same confidence. However, the fitted curve shown in figure 2 at 50 mM NaCl gives values of K_1' as *ca.* 100 μM and k_{2a}' as $13\ s^{-1}$, suggesting that increasing the salt concentration decreases the affinity of GAP for p21.mantGTP and possibly also increases the rate of the conformational change.

7. CONCLUSIONS

Modifications of the ribose moiety of guanine nucleotides with the *N*-methylanthraniloyl group gives analogues which have similar kinetic and equilibrium parameters to the parent nucleotides. The fluorescence changes occurring on the hydrolysis of mantGTP bound to p21 at 30°C, and occurring when p21.mantGMP.PNP is incubated at 30°C, have been used to propose that the rate of the hydrolysis step is controlled by a preceding isomerization step. GAP accelerates this isomerization and hence the overall GTPase, although this does not rule out the possibility that GAP also contributes catalytic groups that enhance the chemical cleavage rate. From these experiments it is not possible to define the nature of the conformational change although it may be related to the different conformations of the residues 58–65 observed in the crystal structure of wild type p21 (Goody *et al.*, this symposium).

It should be noted, however, that Goody *et al.* (this

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symposium) were unable to observe the slow fluorescence change occurring with $p21^{H\text{-}ras}$.mantGMP.PNP and hence any acceleration by GAP. Based on the lack of a signal they concluded that the fluorescence changes were associated with the cleavage step. However, Antonny *et al.* (1991) observed a change in tryptophan fluorescence in a single tryptophan mutant of p21H-ras in its complex with GTPγS which occurs faster than the cleavage of this nucleotide. They interpret their results to show that in the absence of GAP a conformational change precedes cleavage, but as the fluorescence change is not accelerated by GAP, this step is not on the GAP-activated pathway.

The stopped flow data provide detailed information about the rates of elementary processes occurring on GTP hydrolysis in the presence of GAP. They show that under low salt conditions the initial formation of the p21.mantGTP.GAP complex is formed at rates close to the diffusion-controlled limit, and that the subsequent isomerization is increased by a factor of 10^5 compared with that in the absence of GAP. It is not yet known at which stage GAP dissociates from the complex with p21.nucleotide, and therefore which is the predominant pathway for the GAP-activated mechanism shown in scheme 4. Nor is it known whether conformational changes occur in GAP during its interaction with p21. If such changes do occur they might be related to a possible effector function of GAP. A combination of the fluorescence techniques and rapid reaction methods described here is being used to address these problems.

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Discussion

D. R. Trentham (*National Institute for Medical Research, Mill Hill, London, U.K.*). Do the authors have information about the affinity constant of GAP to the GDP form of ras?

J. F. Eccleston. There is a lot of information in the literature that varies over quite a large range, for example, the affinity for GAP of the leucine-61 mutant to which it binds, but GTP is hydrolysed very slowly, the literature values vary from 5 to 100 μM. The values are very sensitive to ionic strength, and different laboratories have used different conditions and temperatures, but GAP probably binds to p21.GDP at least two orders of magnitude weaker than to p21.GTP.

R. N. Thorneley (*AFRC Division of Nitrogen Fixation, University of Sussex, Brighton, U.K.*). Could the authors clarify about dimerization of ras and the concentration range over which it occurs. Does that have any implications for their transient kinetic studies or is it a dimer which has two independently functioning sites?

J. F. Eccleston. There are several reports in the literature showing that p21ras can aggregate into dimers or higher oligomers. We have evidence that it exists as dimers from time-resolved anisotropy measurements. It is very important that this is characterized in more detail to answer exactly the questions you are asking, and to see whether the

phenomenon is responsible for any of the fluorescence changes observed.

R. H. Michell (*Department of Biochemistry, University of Birmingham, U.K.*) I was curious about the emphasis the authors put upon this effect of ionic strength, as ras inside a cell, which is the only place it ever does its job, is in a medium of substantial ionic strength. Is it of any particular help to think about its action and how it works at zero ionic strength?

J. F. Eccleston. Most of the work has been done at higher ionic strength. The reasons the initial stopped flow experiments were done at lower ionic strength are twofold. First, we wanted to make a direct comparison with the high activations reported by others on full length and truncated GAP under these low ionic strength conditions. Second, although equilibrium and rate constants of the GAP activated mechanism are highly dependent on salt conditions, the actual mechanism is unlikely to vary, and only by using low ionic strength could we define the hyperbolic dependence of the process on GAP concentration. The observed *in vitro* reduction of GAP activity at physiological ionic strengths raises important questions about the regulation and level of GAP activity *in vivo*.

K. C. Holmes (*Max-Planck-Institute für medizinische Forschung, Heidelberg, F.R.G.*). I think I should emphasize what Dr Eccleston has said; the nuclear magnetic resonance (NMR) work has shown quite a strong tendency for ras to aggregate, although one does not know whether this is as dimers or trimers, etc. This may be a function of the state of the nucleotide. The crystallization conditions of the GTP and GDP form are completely different, the nature of the crystals are completely different, and the nature of the protein–protein interactions in the crystals is completely different. Protein–protein interactions, after all, are what are important in dimerization.

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