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Expression of p21 proteins in *Escherichia coli* and stereochemistry of the nucleotide-binding site

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v-Ha-*ras* encoded p21 protein ($p21_V$), the cellular c-Ha-*ras* encoded protein ($p21_C$) and its T24 mutant form $p21_T$ were produced in *Escherichia coli* under the control of the *tac* promoter. Large amounts of the authentic proteins in a soluble form can be extracted and purified without the use of denaturants or detergents. All three proteins are highly active in GDP binding, GTPase and, for $p21_V$, autokinase activity. Inhibition of [^{3}H]GDP binding to $p21_C$ by regio- and stereospecific phosphorothioate analogs of GDP and GTP was investigated to obtain a measure of the relative affinities of the three diphosphate and five triphosphate analogs of guanosine. p21 has a preference for the Sp isomers of GDPαS and GTPαS. It has low specificity for the Sp isomer of GTPβS. Together with the data for GDPβS and GTPγS these results are compared with those obtained for elongation factor (EF)-Tu and transducin. This has enabled us to probe the structural relatedness of these proteins. We conclude that p21 seems to be more closely related to EF-Tu than to transducin.

Key words: p21 protein/expression/stereochemistry/nucleotide-binding site

Introduction

The *ras* oncogene family, which includes the Ki-, Ha- and N-*ras* proto-oncogenes, encodes a closely related group of proteins of ~21 000 daltons mol. wt, termed p21 proteins, which are GTP-binding proteins with low GTPase activity (for a review, see Bishop, 1985; Gibbs *et al.*, 1985). They are believed to play an essential role in growth and/or development. A number of structural comparisons and structure predictions have been published in an attempt to explain the structural implications of the amino acid replacements at positions 12, 13 and 59,61, which lead to tumorigenic activation of the protein (Gay and Walker, 1983; Wierenga and Hol, 1983; Leberman and Egner, 1984; Halliday, 1983; Pincus and Brandt-Rauf, 1985; Murakami, 1985). Since secondary structure predictions at the very best are only 60% accurate and are sometimes contradictory to each other, the actual determination of the three-dimensional structure is essential.

Models for the tertiary structure of the nucleotide-binding site of p21 have been built on the basis of partial sequence homologies between elongation factor (EF)-Tu and p21 proteins and using the tertiary structure of the GDP-binding domain of trypsinized EF-Tu from *Escherichia coli* determined by X-ray crystallography (McCormick *et al.*, 1985; Jurnak, 1985). Since crystals suitable for X-ray crystallography have not yet been obtained, we have looked for other means to obtain structural information from the p21 proteins. Thiophosphate analogs of nucleoside di- and triphosphates have been used to study the active site geometry and metal ion coordination in the nucleotide-binding site of many proteins (for a review, see Eckstein, 1985). We have used this method to study the active site of p21 proteins and compared it with results obtained earlier with EF-Tu (Wittinghofer *et al.*, 1982; Leupold *et al.*, 1983); and recently with transducin (Yamanaka *et al.*, 1985). The source of the p21 protein was a bacterial expression system based on the *tac* promoter which produces high amounts of authentic and soluble p21 proteins which are highly active in GTPase and nucleotide binding.

Results

Construction of expression vectors

To express p21 in *E. coli* we used the expression vector pKM-tacI (deBoer *et al.*, 1983) following the scheme outlined in Figure 1. The 880-bp *Hin*dIII fragment of Ha-MuSV was inserted and the correct coding sequence for the p21 protein from v-Ha-*ras* (= $p21_V$) was regenerated by inserting a synthetic linker consisting of two 19-mers. For expression of the *ras* construction the *lac* repressor overproducing strain RRIΔM15 was used. The final clone $ptacras_V$ was identified by immunoblotting the crude extract of the induced culture with anti-*ras* monoclonal antibody Y13-259 (Furth *et al.*, 1982), as shown in Figure 2. The construction was verified by sequencing the plasmid DNA in the region of the translational initiation site.

The normal and the T24 mutant Ha-*ras* proteins were also produced by replacing the 880-bp *Hin*dIII fragment of $ptacras_V$ with the *Hin*dIII-*Sal*I fragment of pSKcHras and pSKT24 (Gross *et al.*, 1985) as shown in Figure 1. These constructs are also transformed into RRIΔM15. They are stably maintained in this strain and the cells can be easily grown, even in large fermenters.

In medium containing high concentrations of ampicillin (100 μg/ml) the *tac* promoter was repressed and very little p21 protein could be identified by SDS-polyacrylamide gel electrophoresis in the crude lysate (see Figure 2). Expression of p21 proteins could be induced with 50-100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) to produce amounts of proteins much greater than 20% of the soluble protein for all three constructions. On SDS-PAGE the viral and the T24 mutant form of *ras* protein have an apparent mol. wt of 23 500 daltons. The cellular form of p21 ($p21_C$) has a slightly higher mobility, which has been observed before by other authors (Fasano *et al.*, 1984; McGrath *et al.*, 1984). A small amount of a 65 000-dalton protein is induced, which also reacts with monoclonal antibody Y13-259 (Furth *et al.*, 1982). The nature of this protein is presently unknown.

Purification of p21 proteins

Lysis of the cell paste and purification by a two-column procedure could be performed essentially by the same method which we have used for the isolation of bacterial EF-Tus and EF-Ts (Leberman *et al.*, 1980), except that after DEAE-Sepharose, AcA54 was used for gel filtration chromatography. No detergent was

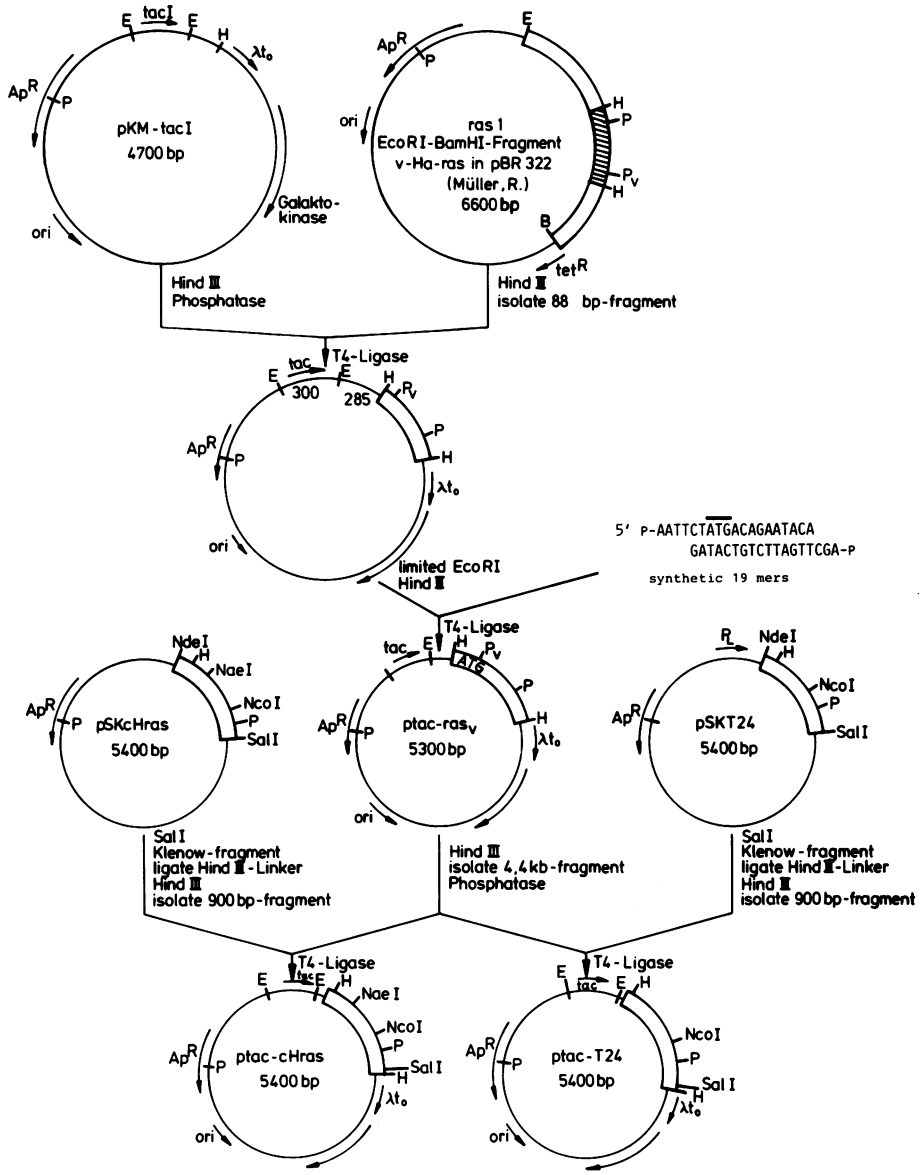

Fig. 1. Construction of the ptacras$_V$ expression vector. Plasmid pRAS1 (R.Mueller, unpublished) containing the 2200-bp *Eco*RI−*Bam*HI fragment of Ha-MuSV (Ellis *et al.*, 1980) was excised with *Hin*dIII, which generates a 880-bp fragment containing the p21 coding region except four codons at the N terminus and 300 bp of 3′-non-coding region (Yasuda *et al.*, 1984). This fragment was ligated into the pKMtac1 expression vector (deBoer *et al.*, 1983) cleaved with *Hin*dIII. The correct orientation of the p21 coding region behind the *tac* promoter was verified by *Pvu*−*Eco*RI double digests. This pre-*ras* plasmid was partially digested with *Hin*dIII and *Eco*RI, the digestion mixture was separated on an agarose gel. The band corresponding to the desired 5300-bp fragment was electroeluted and ligated to the mixture of phosphorylated 19-mers with the indicated sequences, which restored the p21 coding region and the proper distance between ATG start codon and the Shine−Dalgarno sequence (Shine and Dalgarno, 1974). The ligation mixture was transformed into RRIΔM15. The resulting clones were screened by preparing crude lysates from minicultures induced with 50 μM IPTG and analyzing them by SDS−PAGE and immunoblotting. H, E, P, Pv and B denote the restriction enzymes *Hin*dIII, *Eco*RI, *Pst*I, *Pvu*II and *Bam*HI, respectively.

used in the isolation procedure. For analysis of the column effluents the simple GDP exchange assay with nitrocellulose filters was used (Leberman *et al.*, 1980). Figure 3 shows the polyacrylamide gel electrophoresis of crude lysate and the two column effluents for the purification of p21$_C$. The viral and T24 mutant human p21 proteins were purified similarly and lanes 4 and 5 show these two purified proteins. 100 g of cell paste could thus be processed to obtain 320 ± 30 mg of the three proteins as shown in Table I, which gives examples for one preparation of each of the three different p21 proteins. Table I and the polyacrylamide electrophoresis data also show that >20% of the total soluble protein in the cellular extract is isolated as p21 protein with >95% purity. Protein sequencing has been performed for the p21$_V$ protein and the results show that the N terminus of the protein is not blocked, that methionine is the first amino acid and that a unique and expected sequence is obtained as far as arginine in position 12. On SDS−polyacrylamide gels the two transforming, but not the cellular protein occasionally appeared as two bands, both of which reacted with p21 antibody (see Figures 2 and 3). We are presently investigating whether post-translational modification is responsible for this phenomenon.

Characterization of purified proteins

The u.v. spectrum of the purified proteins is identical to that reported for the fusion protein described by Poe *et al.* (1985). We also find that the purified p21 proteins contain one equivalent

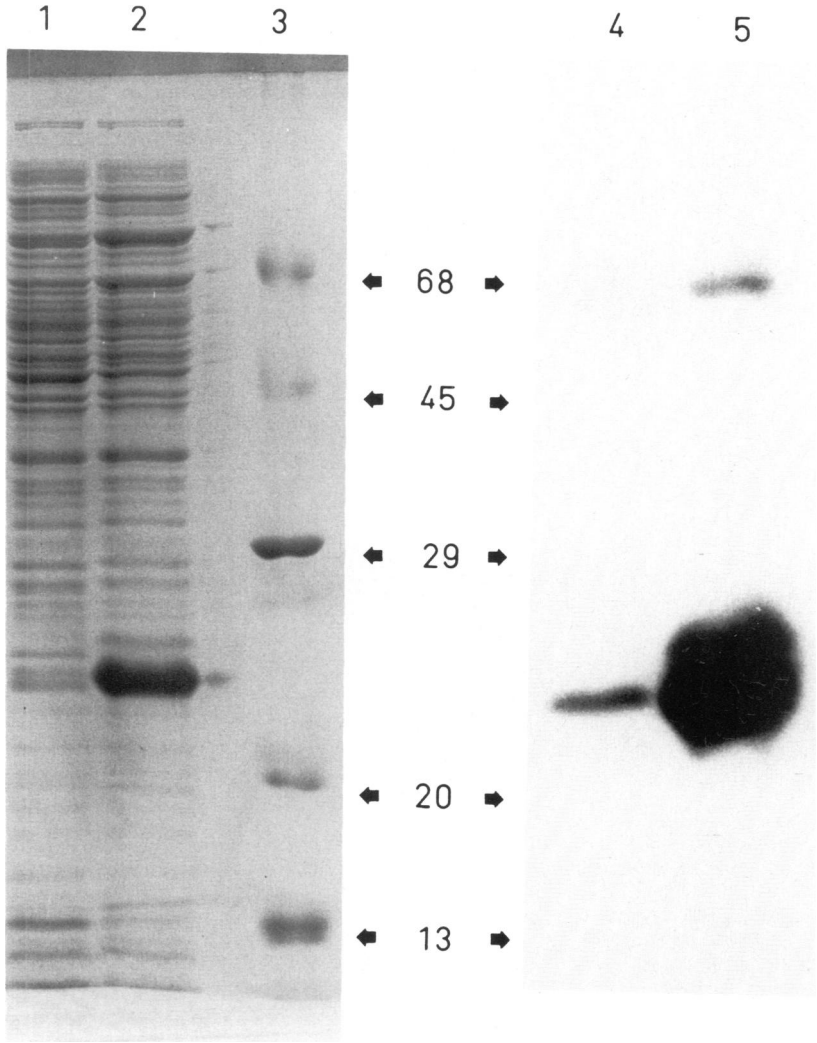

Fig. 2. Induction of $p21_V$ in bacterial cultures. Cells of an overnight culture of $ptacras_V$ in RPIΔM15 in LB medium containing 100 μg/ml of ampicillin in the presence (induced) or absence (uninduced) of 1 mM IPTG were collected. They were washed and a cleared lysate of soluble protein in 100 μl of buffer A was prepared as described in Materials and methods. 25 μl of this solution were treated with SDS sample buffer and separated on a 15% acrylamide gel as described by Laemmli (1970). The lanes were either stained with Coomassie Blue (**lanes 1–3**) or used for immunoblotting (**lanes 4–5**) as described in Materials and methods. The lanes from left to right are: **1**, uninduced; **2**, induced; **3**, mol. wt markers with mol. wts in kd as indicated; **4**, uninduced; **5**, induced.

of bound nucleotide as one would expect for the high binding constants (of the order of 10^8 M^{-1}) reported for the binding of GDP and GTP to p21 (Hattori *et al.*, 1985). Contrary to what has been found by Poe *et al.* (1985), we find that besides GDP (ribo) also deoxy-GDP and a small amount of GTP remain bound to the proteins, which together add to 1 mol of nucleotide per mol of protein (data not shown). 80% of bound nucleotide is GDP only when the elution from the second column (AcA54) is done in the presence of GDP. The p21 proteins are highly active in GDP binding, GTPase activity and the viral protein is also highly active in autophosphorylating activity (see Table II). The GDP binding activity is higher than reported by other authors and is the theoretical maximum – 46 500 pmol bound GDP per mg of protein – if one makes the (reasonable) assumption that 1 mol protein binds 1 mol GDP. The GTPase activity for the purified $p21_c$ is 0.02 min^{-1}, as high as the value reported for the p21 fusion protein (Temeles *et al.*, 1985). Table II also shows that the transforming proteins $p21_V$ and $p21_T$ have a 5- to 8-fold lower GTPase activity, which has been observed by many authors (McGrath *et al.*, 1984; Sweet *et al.*, 1984; Gibbs *et al.*, 1984; Manne *et al.*, 1985). The proteins have also been used for proton and phosphorus magnetic resonance measurements (n.m.r.), where they have been shown to be highly structured, native proteins (Roesch *et al.*, 1986; Roesch *et al.*, unpublished). The proteins also can be crystallized although the crystals obtained are not yet suitable for X-ray crystallography.

Stereochemistry of the nucleotide-binding site

Phosphorothioate analogs of GDP and GTP contain sulfur in place of a non-bridging oxygen atom in one of the phosphorus atoms. Substitution of sulfur for oxygen produces a chiral center at the α-phosphorus of GDP and at the α- and β-phosphorus atoms of GTP. The absolute stereochemistry of the diastereomers is known (see Eckstein, 1985, for references) and they are designated Sp and Rp according to their configuration. Thus there are five regio- and stereospecific thio-GTP analogs: (Sp)-GTPαS, (Rp)-GTPαS, (Sp)-GTPβS, (Rp)-GTPβS, GTPγS, and three thio-GDP analogs: (Sp)-GDPαS, (Rp)-GDPαS and GDPβS, which can be used to probe the active site stereochemistry of p21. Figure 4 illustrates the different phosphate oxygens of GTP which are replaced by

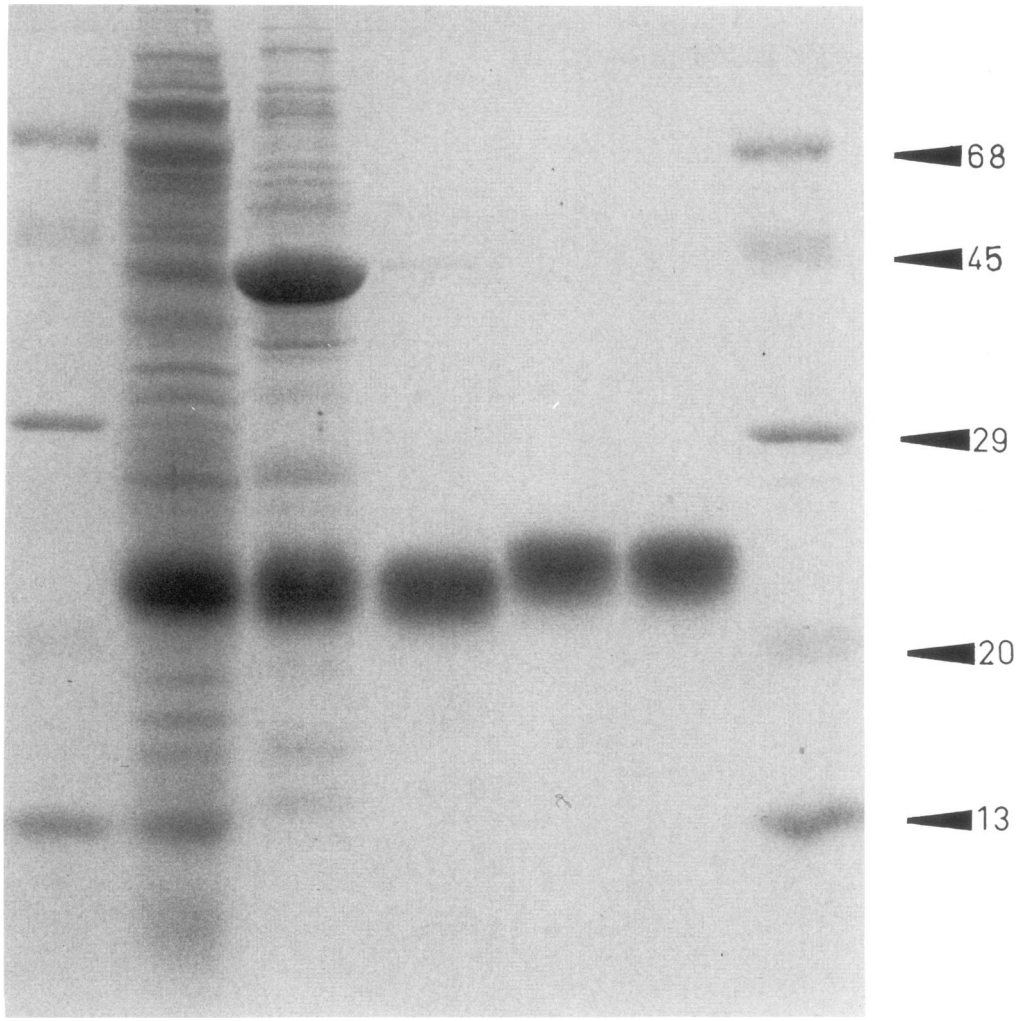

Fig. 3. SDS–PAGE of fractions from the purification of $p21_C$ and purified $p21_V$ and $p21_T$. The lanes from left to right contain: mol. wt makers as indicated on the right, crude lysate, DEAE–Sepharose pool, AcA54 pool, $p21_T$, $p21_V$ mol. wt markers.

sulfur in the thio-GTP analogs. Pro-R and pro-S indicate the oxygen ligands on α- or β-phosphorus, whose substitution by sulfur leads to the corresponding (Rp) and (Sp) isomers, respectively. The diastereomers of GDP(α-S) have structures corresponding to those of GTP(α-S).

We have measured the relative affinity of the phosphorothioates of GDP and GTP by exchanging GDP bound to p21 with these analogs. Figure 5 shows that the exchange reaction between bound GDP and free radioactive [^{3}H]GDP, which is also the basis of the GDP binding activity measurements, is a very slow reaction at room temperature and takes >6 h before equilibrium is reached. Addition of excess EDTA speeds up the reaction considerably so that equilibrium conditions are reached after 15 min. It appears that the metal ion in the p21.GDP.Me^{2+} complex is readily available to solvent. Thus the dissociation of bound GDP, which is probably rate-limiting in the exchange reaction, is increased dramatically after metal ion removal, which has also been observed for EF-Tu (Eccleston, 1981).

Inhibition of [^{3}H]GDP binding was measured with increasing concentrations of thiophosphate analogs. The exchange reaction was performed in the presence of EDTA, but after attainment of equilibrium excess magnesium was added. After incubating for another 30 min the reaction mixture was filtered through nitrocellulose filters and washed. Figure 6 shows as an example the inhibition of GDP binding by (Sp)-GDPαS and (Rp)-GDPαS. $p21_C$ shows a selectivity for the (Sp)-isomer of GDPαS and the relative affinities of the analogs versus GDP can be estimated from the concentrations of analog which cause 50% inhibition

Table I. Summary of protein yields for the purification of c-Ha-*ras* encoded $p21_C$, its T24 mutant forms, $p21_T$ and the v-Ha-*ras* encoded protein $p21_V$, starting with 100 g cell paste

	$p21_C$ (mg)	$p21_T$ (mg)	$p21_V$ (mg)
Crude	1591	1319	1486
DEAE	915	870	889
ACA54	350	318	325

Table II. Biochemical activities of the three purified p21 proteins

Activity	Protein		
	$p21_C$	$p21_V$	$p21_T$
GDP-binding (U/mg)	46 500	45 100	45 600
GTPase (min^{-1})	0.02	0.005	0.0025
Autokinase (min^{-1})	–	3.8×10^{-4}	–

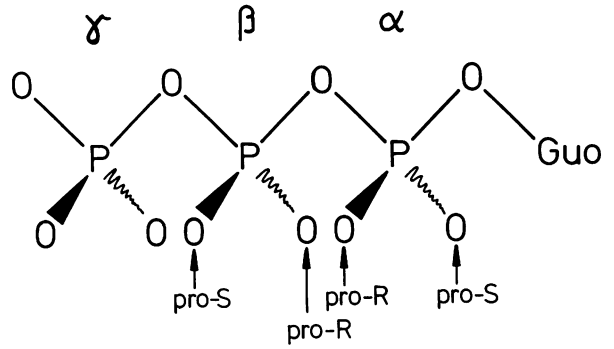

Fig. 4. Schematic drawing showing the oxygens of the α-, β- and γ-phosphates of GTP. The α- and β-phosphorus atoms are pro-chiral centers, so that substitution of one of the non-bridging oxygens (designated pro-R) by sulfur leads to a chiral center with the Rp absolute configuration, and substitution of the other (pro-S) to the Sp configuration. The diastereomers of GDP(α-S) have the same configuration as those of GTP(α-S). Substitution of one of the three γ-phosphate oxygens does not create a chiral center.

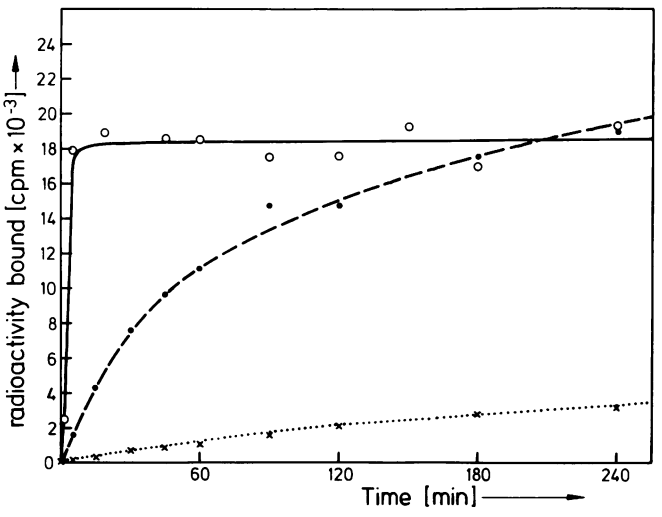

Fig. 5. Kinetics of the GDP exchange reaction. 25 μM p21 was incubated with 10 μM [^{3}H]GDP in the presence or absence of 0.6 mM EDTA in a final volume of 1 ml. At the indicated times, 50 μl aliquots were removed and the amount of [^{3}H]GDP bound to protein was determined as described in Materials and methods.

of GDP binding. Thus 8 μM (Sp)-GDPαS and 54 μM (Rp)-GDPαS cause 50% inhibition, and the ratio of affinities of the (Sp)- and (Rp)- isomers (Sp/Rp) is 6.5. The 50% inhibition values are used to obtain the relative affinities for all the thiophosphate analogs as shown in Table III. If we take the binding constant for p21.GDP as unity [its absolute value is 1.0×10^8 M^{-1} as determined by Hattori *et al.* (1985)], we find relative binding constants for (Sp)-GDPαS of 1.3 and 0.2 for the (Rp)-isomer, which means that (Sp)-GDP(αS) binds better than GDP. Using the same method, we find that the affinity of GTP is higher than GDP by a factor of 1.4, which agrees well with what has been found by Finkel *et al.* (1984), Hattori *et al.* (1985) and Manne *et al.* (1984), who have reported that the two nucleotides bind with similar affinities. (Sp)-GTP(αS) has a relative affinity of 0.6, i.e. it binds more weakly than either GDP or GTP. The relative affinities for the GTPαS diastereomers (Sp/Rp) are of the same order but the relative strength is 12.6 as compared with 6.5 for the GDPαS diastereomers. Both GTPβS isomers have

Table III. Relative affinities of phosphorothioate analogs of GDP and GTP to p21, and to EF-Tu (Wittinghofer *et al.*, 1982; Leupold *et al.*, 1983) and transducin (Yamanaka *et al.*, 1985) as taken from the literature

Analog		Protein		
		$p21_C$	EF-Tu	Transducin
GDP[αS]	Sp	1.3	0.23	4.0
	Rp	0.2	0.0083	0.6
	Sp/Rp	6.5	27.0	6.7
GDP[βS]		0.36	0.041	0.4
GTP[αS]	Sp	0.6	0.29[a]	–
	Rp	0.05	0.015[a]	10
	Sp/Rp	12.0	19.3[a]	–
GTP[βS]	Sp	0.016	0.0103[a]	0.83
	Rp	0.019	0.0108[a]	$<10^{-2}$
	Sp/Rp	0.84	0.95[a]	>83
GTP[γS]		0.29	0.36[a]	>10
GTP		1.5	0.01[a]	

The relative affinities of analogs is the inverse of the ratio of concentration [analog]/[GDP] which causes 50% inhibition of GDP binding, as demonstrated for the GTPαS analogs in Figure 4 (not shown for the other analogs). The affinity of $p21_C$ for GDP is defined as one.
[a]These values were obtained by measuring the inhibition of the GTPase reaction with varying concentrations of GTP analogs. The 50% inhibition value is thus not a measure of relative affinity versus GDP.
[b]Miller and Weissbach (1970).

Table IV. Turnover number for the guanosine triphosphatase reaction between $p21_C$ and GTP and phosphorothioate analogs of GTP, determined with 0.1 mM protein and 1 mM nucleotide as described in Materials and methods

Substrate	Turnover number (min^{-1})
GTP	0.02
(Sp)-GTPαS	0.005
(Rp)-GTPαS	0.004
(Sp)-GTPβS	0.0005
(Rp)-GTPβS	0.002
GTPγS	–

relatively low affinities for p21 and the association constant is lower by a factor of 52–62, but there is a slight preference for one diastereomer, (Rp), which is favored over the (Sp)-isomer by a factor of 1.2. The substrate activity of the GTP analogs was also tested and the results are shown in Table IV. The diastereomers of GTP(α-S) are cleaved at similar rates, and this is considerably lower than that for GTP. The diastereomers of GTP(β-S) are cleaved even more slowly, but with a clear preference for the Rp isomers.

Discussion

In this report we describe the construction of an *E. coli* expression system which can be used to produce high amounts of three different p21 proteins in soluble form. Several reports have appeared describing the use of different expression vectors to produce p21 proteins in *E. coli*. Except in one case (Gross *et al.*, 1985), they all lead to the expression of fusion proteins or insoluble forms of the protein which need considerable amounts of chaotropic salts or detergents to be solubilized. From the results presented here and those of Gross *et al.* (1985) it is apparent that the native protein is highly soluble. The use of denaturing

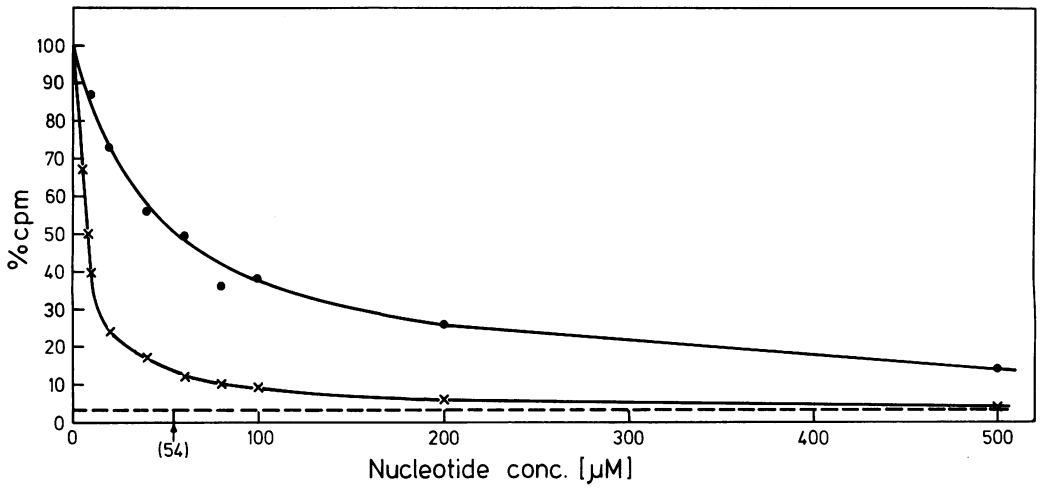

Fig. 6. Inhibition of [^{3}H]GDP binding to $p21_C$ by GDPαS analogs. 2.2 μM $p21_C$ was incubated with 9 μM [^{3}H]GDP and varying concentration of (Rp)-GPDαS (.) and (Sp)GDPαS (x) with excess EDTA. After 150 min at 0°C excess Mg^{2+} was added and the bound [^{3}H]GDP was determined as described in Materials and methods.

agents or detergents may in fact perturb the native protein conformation and may change its biochemical and biological characteristics. The protein described here is highly soluble even at the concentrations (~30 mg/ml) used for n.m.r. measurements (Roesch *et al.*, 1986). It is produced in *E. coli* in such high amounts that the purification of gram amounts of protein for structural studies is relatively easy.

In earlier work we investigated the stereochemistry of the GDP- and GTP-binding site of bacterial EF-Tu by using phosphorothioates of GDP and GTP together with e.p.r. and n.m.r. measurements (Wittinghofer *et al.*, 1982; Leupold *et al.*, 1983; Kalbitzer *et al.*, 1984). This enabled us to propose a model for interactions at the nucleotide-binding site which was later confirmed by the available X-ray data (LaCoeur *et al.*, 1985). The X-ray coordinates were then used to build a model of p21 (McCormick *et al.*, 1985; Jurnak, 1985).

p21, as can be seen from the data in Table III, has a preference for the (Sp)-isomer of GDPαS and GTPαS and this is also true of EF-Tu (Leupold *et al.*, 1983; data shown in Table III). In case of EF-Tu, it was shown that there is no interaction between the α-phosphate group and the metal ion bound with the nucleotide, which leads to the conclusion that the specificity for the Sp diastereomers of GDPαS and GTPαS is due to an energetically important interaction of the protein with the pro-R oxygen of this phosphate group and it is likely that this is also true for p21. We expect an unambiguous clarification of this feature from our current e.p.r. experiments with p21.

There is very little specificity for the binding of the diastereomers of GTPβS, again in agreement with the results obtained with EF-Tu, and in contrast to the high Sp/Rp ratio seen for transducin (Yamanaka *et al.*, 1985; data shown in Table III). In the case of p21, it was possible to measure the rate of cleavage of the GTP(β-S) diastereomers directly, and this showed a clear preference for the Rp isomer. A common feature for all three proteins, and indeed for many others studied, is that the replacement of either oxygen of the β-phosphate group by sulfur leads to a large reduction in affinity, suggesting that both oxygens are involved in important interactions, in general one being bound to the metal ion and one to the protein. EF-Tu shows a reversal of specificity (Rp with Mg^{2+}, Sp with Cd^{2+}) for the diastereomers of GTPβS when Mg^{2+} is replaced by Cd^{2+}, which is a strong indication that there is an interaction between the pro-S oxygen of the β-phosphate group and the metal ion (Leupold *et al.*, 1983). We have recently confirmed this interpretation by e.p.r. spectroscopy (Kalbitzer *et al.*, in preparation). Again, our current e.p.r. experiments should give a definitive answer to this question for p21, but the preference for the Rp diastereomer of GTP(β-S) in the cleavage reaction suggests that here there is also an interaction of the metal ion with the pro-S oxygen of the β-phosphate group. In contrast with EF-Tu, there is no metal ion dependence of the specificity of transducin for the diastereomers of GTPβS (Yamanaka *et al.*, 1985). As suggseted by the authors, this indicates either that there is no interaction of the metal ion with the β-phosphate group or that a very strong interaction with the protein forces the metal to bind to the same residue (sulfur) regardless of the nature of the metal ion. Assuming the latter interpretation to be correct, this leads to the initially unexpected conclusion that the pro-S oxygen (see Figure 4) is bound to the metal ion, as it is the case for EF-Tu, despite the seemingly contrasting properties with respect to the specificity for the diastereomers.

From the results presented and discussed here together with the cited work on EF-Tu and transducin, we can conclude that there are similarities in the active site stereochemistry between p21, EF-Tu and transducin with respect to both the α- and β-phosphate groups. The similarity between the interactions of the β-phosphate groups of GTP with EF-Tu and with p21 is probably greater than that between the interactions of either of these proteins with transducin, although this cannot be maintained with certainty until the e.p.r. results for p21 are available. It should be pointed out that many other nucleoside triphosphatases and kinases (e.g. myosin and adenylate kinase; Hofmann and Goody, 1980; Connolly *et al.*, 1982) have been shown to have an interaction between the metal ion and the pro-R oxygen atom of the β-phosphate group, suggesting a different geometry around this phosphate when compared with the GTP-binding proteins discussed here.

Both GDPβS and GTPγS, for which diastereomers are not formed by the replacement of oxygen by sulfur, are fairly good analogs for GDP and GTP. In this respect, p21 is more similar to transducin than to EF-Tu, since GDPβS is a poor analog of GDP for ET-Fu (see Table III). This only applies to EF-Tu in

the presence of Mg^{2+} since, in the presence of excess EDTA, GDPβS is a good analog (Wittinghofer *et al.*, 1982). This has led us to suggest that in the EF-Tu.MgGDP complex all three non-bridging β-phosphate oxygens are involved in important interactions, one with metal ion and two with the protein. An extension of the same arguments leads to the conclusion that this does not apply to p21 or transducin, i.e. that in these complexes perhaps only one of the β-phosphate oxygens is bound to protein.

Both EF-Tu and transducin are guanosine triphosphatases, and the interconversion between the GTP and GDP bound states is coupled to their translocation, either to and from the ribosome (Kaziro, 1978), or to and from the cyclic-GMP phosphodiesterase in the membrane, which amplifies the signal of transducin (Stryer, 1983). In the case of *ras* proteins it is postulated, and it has been shown for the yeast *ras* proteins, that they stimulate membrane-bound adenylate cyclase (Toda *et al.*, 1985; DeFeo-Jones *et al.*, 1985). It is thus of interest to look for similarities and differences between the proteins. On the basis of the results on p21 presented here and the published work on EF-Tu and transducin, there are some obvious similarities but also quantitative differences. Amongst the similarities are the very low GTPase activities of the isolated proteins (i.e. when they are not interacting with the other components of their *in vivo* systems), the high binding affinities for guanosine nucleotides and certain stereochemical similarities of their interactions with nucleotide and metal ions discussed above. A quantitative comparison suggests that there is perhaps more similarity between p21 and EF-Tu than between p21 and transducin or EF-Tu and transducin, although in one respect, i.e. the interaction of the β-phosphate of GDP with metal ion and protein, p21 and transducin are similar to each other but significantly different from EF-Tu, as discussed above. The models presented for the structure of the nucleotide-binding site of p21 (McCormick *et al.*, 1985; Jurnak, 1985) might be good aproximations of the real three-dimensional structure, although the results discussed here show that there are obviously differences in detail.

In considering the similarities of the three proteins, the question of the function of p21 arises, since it has been shown that the RAS1 and RAS2 gene products of yeast, which regulate adenylate cyclase activity, are highly homologous to mammalian *ras* gene products, at least in their N-terminal 180 amino acids (Dhar *et al.*, 1984; Powers *et al.*, 1984). Thus it has been postulated that these proteins have an analogous function to that which G-proteins and transducin have in the amplification of extracellular signals. The human Ha-*ras* oncogene product, however, is not a component of adenylate cyclase (Beckner *et al.*, 1985) and it does not seem to be specifically ADP-ribosylated. In addition, in a recent report by Birchmeier *et al.* (1985) it was shown that human Ha-*ras* p21 proteins injected into oocytes induce maturation but do not change the cyclic AMP concentration. These results and our analysis of the relatedness of the active site geometries suggest that the mammalian *ras* proteins are perhaps functionally different from the G-proteins and transducin.

Materials and methods

[^{3}H]GDP (400 GBq/mmol) was obtained from Amersham Buchler and was diluted to the desired specific activity with unlabelled GDP (Pharma Waldhof). Phosphorothioate analogs of GDP and GTP were synthesized according to Goody and Leberman (1979) and Roesch *et al.* (1984) except that glycerol kinase was used to remove traces of (Rp)-GTPβS from (Sp)-GTPβS as described by Connolly *et al.* (1982). DEAE−Sepharose CL-6B was from Pharmacia and AcA54 from LKB. IPTG was from Serva Heidelberg. Nitrocellulose filters, type BA85, 0.45 μM were from Schleicher and Schuell. H.p.l.c. grade acetonitrile was obtained from Baker. All other reagents were of the highest purity available.

Plasmids and strains

Plasmid pras1 contains the 2200-bp *Eco*−*Bam*HI fragment of Ha-MuSV cloned between *Eco*RI and *Bam*HI of pBR322 (R.Mueller, unpublished). pKM-tac1 (deBoer *et al.*, 1983) and the two 19-mers (see Figure 1) were a kind gift of A.Ullrich. RRIΔM15 [leu,pro,thi,lacΔM15,r-,m-,F'lacI^{Q}Z,ΔM15,pro$^+$] is a *lac* repressor overproducing strain derived from HB101 (Boer and Roulland-Dussoix, 1969), which was obtained from B.Mueller-Hill. Plasmids pSKcHras and pSKT24 (Gross *et al.*, 1985) were obtained from O.Fasano.

Cloning methods

Restriction endonucleases, alkaline phosphatase, T4 ligase and polynucleotide kinase were commercial products of Boehringer Manhheim or BRL and were used following the Laboratory Manual of Maniatis *et al.* (1982). Transformation was done according to the method of Hanahan (1983) with frozen cells. The sequence of the final ptacras$_V$ construction was verified by sequencing according to the method of Maxam and Gilbert (1977).

Cell-free extract and immunoblotting

A 1 ml miniculture of ptacras$_V$ in RRIΔM15 was grown in LB medium containing 30 μg/ml of ampcillin. 10 μl of this culture was used as an inoculum for a fresh 1 ml culture in LB medium with 100 μg/ml of ampicillin in the presence and absence of 100 μM IPTG. After growing the cells overnight at 37°C, they were collected by centrifugation for 10 s in an Eppendorf centrifuge, washed with 10 mM Tris-HCl pH 7.4 and lysed basically by scaling down the procedure of Leberman *et al.* (1980). The crude cell extract was run on a 15% SDS−PAGE essentially as described by Laemmli (1970). One part of the gel was stained with Coomassie Blue, the other was blotted onto nitrocellulose filters and reacted with the anti-p21 antibody Y13-259 (Furth *et al.*, 1982) and with a second anti-rat antibody as described by Yamamoto *et al.* (1985).

Cell culture

4 l of an overnight culture of RRIΔM15 containing the appropriate plasmid in LB-medium containing 50 μg of ampicillin/ml were used as an inoculum for 80 l of rich medium (in g/l:Difco casamino acids, 10; Difco yeast extract 7.5; $KH_2PO_4.H_2O$, 5; K_2HPO_4, 5; $MgSO_4.7H_2O$, 1; glycerol, 18; ampicillin 0.1). Growth was carried out at 37°C to an A_{260} of 1.5 and then the culture was induced with 50 μM IPTG for 8 h at 37°C. The cells were harvested by centrifugation to give a 2000 *g*−2400 *g* cell paste, which was stored at −70°C.

Isolation procedure

105 g of frozen cell paste was homogenized with 500 ml of buffer A [64 mM Tris, 50 mM HCl, 1 mM NaN_3, 0.5 mM dithioerythritol (DTE), pH 7.6 at 4°C]. Phenylmethylsulfonayl fluoride (PMSF) to 0.01 mM, benzamidine to 0.01 mM, 1 ml 0.5 M EDTA pH 7.6 and 200 mg of lysozyme were added and the suspension re-homogenized. After 30 min at 4°C 5 ml of 1 M $MgCl_2$, 2.5 ml of 4% sodium deoxycholate and 20 mg of DNase I were added. After a further 30 min of incubation at 4°C the viscosity of the suspension had decreased and it was then centrifuged at 12 000 r.p.m. for 30 min in a Sorvall GSA rotor at 4°C. The supernatant, 540 ml, was applied to a column of DEAE-Sepharose CL-6B (5 × 27 cm) equilibrated in buffer B (buffer A containing 10 mM $MgCl_2$). The column was washed with 500 ml of buffer B and then developed with a 5000 ml linear gradient of 0−0.4 M NaCl in buffer B. The flow-rate was 3.6 ml/min and 20-ml fractions were collected. Fractions containing p21 (as detected by the GDP exchange assay and by SDS−PAGE) were pooled and brought to 60% saturation with ammonium sulfate. After 20 min the precipitate was recovered by centrifugation, dissolved in 15 ml of buffer B, clarified by centrifugation and applied to a system of two consecutive columns of AcA54 (2.5 × 200 cm) in buffer C (buffer B containing 0.1 mM GDP and 200 mM NaCl). The column was developed with buffer C at a flow-rate of 1.2 ml/min and 9-ml fractions were collected. The second major peak of GDP exchange activity was located and fractions free from impurities (as detected by SDS gel electrophoresis) were pooled and brought to 70% saturation with ammonium sulfate. The precipitated protein was collected by centrifugation, dissolved in 20 ml buffer B and dialyzed against buffer B containing 10 mM DTE overnight. All purification procedures were carried out between 0 and 4°C.

H.p.l.c.

A C-18 reversed-phase column (0.4 × 25 cm) filled with Shandon Hypersil 5 μ was obtained from Abimed. The system consisted of a Beckman 280B pump and a u.v. III-detector (252 nm) from Latek. The column was run at ambient temperature with a flow-rate of 2.5 ml/min with phosphate buffer (50 mM, pH 6.5) containing 0.2 mM tertiary butylammoniumbromide, 3% (v:v) acetonitrile and 0.2 mM NaN_3. In this system the order of elution is GMP, GDP and GTP (retention times 2.14, 3.67 and 6.02 min, respectively). The amount and identity of nucleotide bound to p21 could be determined by applying the protein sample directly to this column and eluting with the buffer. The column was calibrated with solutions of appropriate nucleotides of known concentrations.

[^{3}H]GDP exchange reaction

For monitoring the column effluents during the isolation procedure the [^{3}H]GDP exchange assay as described previously for EF-Tu was used (Leberman *et al.*, 1980). Maximum exchange of protein bound GDP against [^{3}H]GDP (200–300 c.p.m./pmol) was achieved by incubation of 0.2–2 μM p21 and 8–10 μM [^{3}H]GDP in buffer A for 30 min at room temperature in the presence of 0.6 mM EDTA. The incubation mixture is filtered through nitrocellulose filters and the filter-bound radioactivity determined.

Inhibition studies

2.2 μM $p21_C$, 9 μM [^{3}H]GDP and varying concentrations of analog with 0.6 mM EDTA were incubated for 2.5 h at 0°C in a total volume of 50 μl. 1 μl of 0.5 M $MgCl_2$ was added and the mixture was incubated for 30 min. The probes were filtered and the filter-bound radioactivity determined.

Determination of protein concentration

Protein concentration was assayed routinely the method of Bradford (1976) and in some cases by the method of Lowry *et al.* (1950) using bovine serum albumin (BSA) for calibration. Both methods gave the same results. The values obtained agreed well with the results obtained from the [^{3}H]GDP exchange assay assuming a 1:1 complex between p21 and GDP (Poe *et al.*, 1985). The amount of GDP bound was also determined by h.p.l.c. using a calibration curve obtained with a GDP solution of known concentration. Assuming a 1:1 complex between nucleotide and protein, this led to the same value for the protein concentration as that obtained by the method of Bradford (1976). We could thus determine a molar absorption coefficient for the complex of E_{280} = 18 450 M/cm at ambient temperature. The value for E_{280} is higher than the one published by Poe *et al.* (1985).

GTPase assay

GTPase was assayed by using the calibrated h.p.l.c. system as described above. 5 μl of the reaction mixture (0.1 mM p21 and 1 mM nucleotide in buffer B) were injected onto the h.p.l.c. column after incubation for various times at 37°C and analyzing for the decrease of guanosine triphosphate or the increase in guanosine diphosphate concentration.

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