Biochemical properties of the *ras*-related *YPT* protein in yeast: a mutational analysis

P.Wagner, C.M.T.Molenaar, A.J.G.Rauh[1], R.Brökel, H.D.Schmitt and D.Gallwitz

Max-Planck-Institut für biophysikalische Chemie, Abteilung Molekulare Genetik, Am Fassberg, D-3400 Göttingen, FRG

[1]Present address: Max-Planck-Institut für Molekulare Genetik, D-1000 Berlin-Dahlem, FRG

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Using site-directed mutagenesis, the *ras*-related and essential yeast *YPT1* gene was changed to generate proteins with amino acid exchanges within conserved regions. Bacterially produced wild-type proteins were used for biochemical studies *in vitro* and were found to have properties very similar to mammalian *ras* proteins. Gene replacement allowed the study of physiological consequences of the mutations in yeast cells. $Lys^{21} \rightarrow$ Met and $Asn^{121} \rightarrow$ Ile substitutions rendered the protein incapable of binding GTP and caused lethality. $Ser^{17} \rightarrow$ Gly and $Ala^{65} \rightarrow$ Thr substitutions slightly changed the protein's apparent binding capacity for either GDP or GTP and altered its intrinsic GTPase activity. These mutations were without effect on cellular growth. The $YPT^{gly17,thr65}$ mutant protein displayed a significantly altered relative capacity for guanine nucleotide binding but a GTPase activity comparable to the wild-type protein. In contrast to the $Ala^{65} \rightarrow$ Thr substitution, the double mutant displayed a significantly reduced capacity for autophosphorylation and allowed cells to grow only poorly. Cellular growth was improved when this mutant protein was overproduced.

Key words: yeast *YPT1* gene/site-directed mutagenesis

Introduction

Several cellular proteins have been shown to exert their biological function via a transient binding of guanine nucleotides. Usually, the GTP-bound form of these proteins is active and an intrinsic GTPase activity is prerequisite for the hydrolysis of GTP to GDP and inorganic phosphate and the subsequent GDP−GTP exchange reaction. The number of GTP-binding regulatory proteins seems to be large, the best known examples being the G proteins (see Gilman, 1984, for review) and *ras* proteins (see Gibbs *et al.*, 1985, for review). In eukaryotic cells the G protein genes and the *ras* genes constitute families of genes coding for a still growing number of closely related proteins.

The *ras* proteins, besides their ability to bind and hydrolyze GTP, possess autophosphorylating activity when an alanine residue in position 59 is mutated to become a threonine residue (Shih *et al.*, 1980, 1982). In mammalian cells *ras* proteins are bound to the plasma membrane, very likely through a covalently attached palmitic acid (Willumsen *et al.*, 1984).

A large body of evidence suggests that *ras* proteins are potentially oncogenic, and these findings have led to an intensive study of these proteins. The normal cellular function of *ras* proteins, however, has not been disclosed.

The discovery in yeast of genes with significant homology to the mammalian *ras* proteins (Gallwitz *et al.*, 1983; DeFeo-Jones *et al.*, 1983; Powers *et al.*, 1984) raised the hope that, with the help of sophisticated genetic analyses, the cellular function of *ras* proteins might come to light, at least in this unicellular organism. Five *ras*-related genes, *YPT1*, *RAS1* and *RAS2*, *RHO1* and *RHO2*, have been discovered in the yeast *Saccharomyces cerevisiae*. It has been shown that the protein products of two of them, *RAS1* and *RAS2*, are essential and regulate adenylate cyclase (Toda *et al.*, 1985). The 23.5 kd protein product of the *YPT1* gene also has an essential function in yeast, but in contrast to the *RAS1* and *RAS2* proteins, it might function in microtubule organization and mitosis (Schmitt *et al.*, 1986). Nothing is known so far about the protein products of the recently described *RHO* genes except that the *RHO1* gene serves an essential function (Madaule *et al.*, 1987).

In this report we present our investigations on the biochemical properties of the *YPT* protein. We have introduced several mutations into the *YPT1* gene leading to mutant proteins with altered capacities for GTP binding and hydrolysis and for autophosphorylation. Our studies show that the yeast *YPT* protein shares very similar biochemical properties with the mammalian *ras* proteins.

Results

The YPT protein binds guanine nucleotides

The prominent biochemical properties of the *ras* proteins are their ability to bind and hydrolyze GTP (see Gibbs *et al.*, 1985, for review). The *YPT* protein shares significant homology with the *ras* gene products and other GTP-binding proteins in regions which are believed to participate in nucleotide binding and GTPase activity (Figure 1). We have previously shown that the *YPT* protein formed in yeast cells binds GTP (or dGTP) specifically (Schmitt *et al.*, 1986).

Guanine nucleotide binding could also be observed with the *YPT* protein expressed in *E. coli*. For the bacterial expression we introduced, by site-directed mutagenesis, *Nde*I restriction sites at the start of the protein coding regions of the *YPT1* gene and the *lacZ* gene such that the ATG of the *Nde*I recognition sequence 5′-CATATG-3′ served as the translation initiation codon. This manipulation allowed us easily to insert the wild-type *YPT1* gene and its mutated versions into a modified pUC vector and to express the yeast proteins in *E. coli* under the transcriptional control of the *lac* promoter and without the disadvantage of producing hybrid proteins.

We found that in *E. coli* strain JM 101 the intact *YPT* protein was readily soluble and amounted to about 1−2% of the total cellular protein after 3 h of induction with IPTG. Initially, after cell lysis with detergent-containing buffer according to Laemmli (1970), the bacterial proteins were separated by SDS−PAGE, electrophoretically transferred to nitrocellulose filters and GTP binding was probed on the filters. This method has also been used by McGrath *et al.* (1984) to compare the extent of GTP
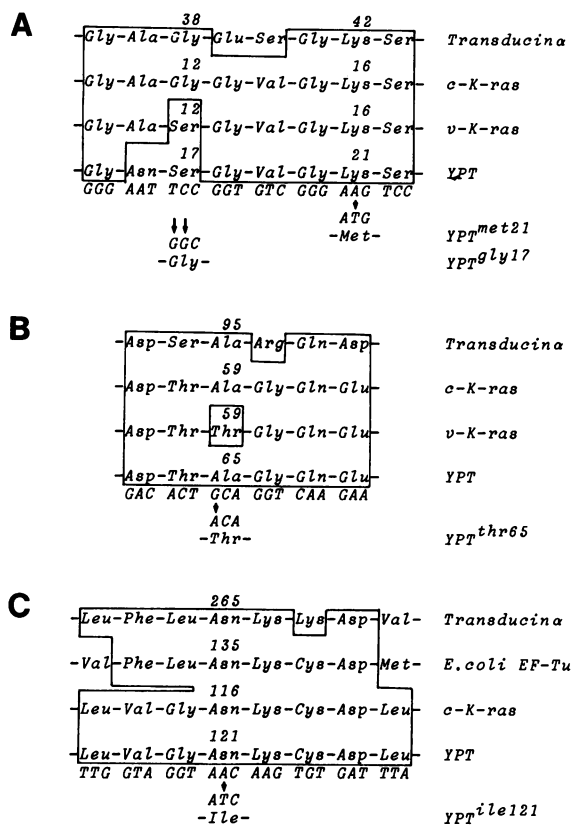
A

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        38                    42
-Gly-Ala-Gly Glu-Ser Gly-Lys-Ser    Transducin α
        12                    16
-Gly-Ala-Gly-Gly-Val-Gly-Lys-Ser-   c-K-ras
        12                    16
-Gly-Ala-Ser Gly-Val-Gly-Lys-Ser-   v-K-ras
        17                    21
-Gly-Asn-Ser Gly-Val-Gly-Lys-Ser-   YPT
GGG AAT TCC GGT GTC GGG AAG TCC
         ↓↓              ↑
         GGC            ATG
        -Gly-          -Met-         YPT^met21
                                     YPT^gly17
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B

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        95
-Asp-Ser-Ala Arg Gln-Asp-           Transducin α
        59
-Asp-Thr-Ala-Gly-Gln-Glu-           c-K-ras
        59
-Asp-Thr-Thr Gly-Gln-Glu-           v-K-ras
        65
-Asp-Thr-Ala-Gly-Gln-Glu-           YPT
GAC ACT GCA GGT CAA GAA
         ↑
        ACA
        -Thr-                        YPT^thr65
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C

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        265
-Leu-Phe-Leu-Asn-Lys Lys Asp Val-   Transducin α
        135
-Val Phe-Leu-Asn-Lys-Cys-Asp Met-   E.coli EF-Tu
        116
-Leu-Val-Gly-Asn-Lys-Cys-Asp-Leu-   c-K-ras
        121
-Leu-Val-Gly-Asn-Lys-Cys-Asp-Leu-   YPT
TTG GTA GGT AAC AAG TGT GAT TTA
         ↑
        ATC
        -Ile-                        YPT^ile121
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Fig. 1. Comparison of three regions conserved in different guanine nucleotide-binding proteins. Identical amino acid residues are boxed. The nucleotide sequence of the *YPT* gene is shown to indicate the changed nucleotides for the generation of the different mutant proteins. Sequence data are from Tanabe *et al.* (1985; transducin α); McGrath *et al.* (1983; C-K-*ras*); Tsuchida *et al.* (1982; v-K-*ras*); Arai *et al.* (1980); *E. coli* EF-Tu); and Gallwitz *et al.* (1983; *YPT*).

binding of bacterially produced normal and activated human *ras* proteins. Using this procedure we observed that the wild-type *YPT* protein as well as several of the mutant proteins retained their GTP-binding property (Figure 2). As can be seen in the same figure, lane 1, there was no indication for a comparable GTP-binding protein in extracts from bacteria that had been transformed with the expression vector alone.

Ras proteins produced in *E. coli* are isolated as a rather stable GDP-protein complex (Poe *et al.*, 1985; Tucker *et al.*, 1986; Hall and Self, 1986). In the presence of Mg^{2+} the GDP is only very slowly exchanged with exogenously added GTP or GDP, but after complexing Mg^{2+} with EDTA this exchange is dramatically increased (Tucker *et al.*, 1986; Hall and Self, 1986). We found the same to be true for the bacterially produced *YPT* protein which was purified by a two-step procedure (see Materials and methods) in the presence of 10 mM Mg^{2+}. As shown in Figure 3A, under conditions of low Mg^{2+} (1 mM Mg^{2+}, 2 mM EDTA, final concentrations) the exchange of GDP against [³H]GDP was complete in about 20 min at room temperature. For the exchange reaction to be complete at 0°C under the same conditions, a time of about 4 h was required using either [³H]GDP or [³²P]GTP (data not shown). There was no difference with respect to the kinetics of the GDP exchange reaction between the wild-type and mutant *YPT* proteins (Ala^{65} → Thr and Ser^{17} → Gly substitutions and the double mutant).

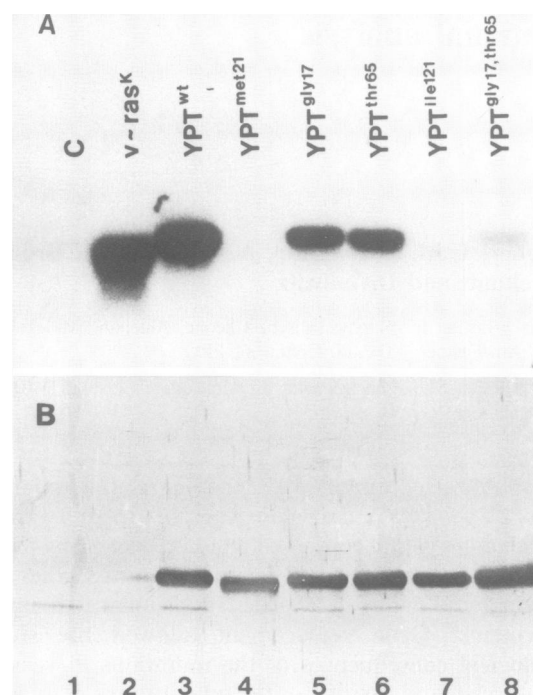Assuming a 1:1 complex between nucleotide and protein, the

Fig. 2. Guanine nucleotide binding of bacterially produced, wild-type and mutant *YPT* proteins. Total cellular protein of *E. coli* JM101, transformed with a pUC8-derived expression vector (**lane 1**, C-control) or the same vector carrying either the viral K-*ras* gene (**lane 2**), the wild-type *YPT1* gene (**lane 3**) or different mutant *YPT1* genes with the substitutions Lys^{21} → Met (**lane 4**), Ser^{17} → Gly (**lane 5**), Ala^{65} → Thr (**lane 6**), Asn^{121} → Ile (**lane 7**), and Ser^{17} → Gly/Ala^{65} → Thr (**lane 8**) was solubilized by 5-min boiling SDS-containing buffer, separated by SDS–PAGE, and transferred to nitrocellulose filters. One filter (**B**) was immunoperoxidase-stained with an antibody against the *YPT* protein. An identical filter (**A**) was treated as described in Materials and methods to analyse the binding of [α-³²P]GTP by the different proteins. Note the reduced apparent affinity for GTP of the mutant proteins YPT^{gly17}, YPT^{thr65}, and $YPT^{gly17,thr65}$ and the lack of GTP binding of the mutant proteins YPT^{met21} and YPT^{ile121} (**lanes 4** and **7**). Also note that the *YPT* antibody does not react with the viral K-*ras* protein.

GDP exchange reaction was used to determine the percentage of active protein, that is the amount of protein in different preparations being able to bind GDP. About 80 to 90% of the proteins isolated with 10 mM Mg^{2+} were active whereas in the absence of Mg^{2+} during the purification procedure the amount of active protein was greatly reduced.

As can be seen in Figure 3B, the nucleotide exchange reaction at room temperature in the presence of 10 mM Mg^{2+} was slow and could be used to compare the relative binding capacities for GDP of the wild-type and mutant *YPT* proteins.

YPT mutations that interfere with the protein's capacity to bind guanine nucleotides

As outlined in Figure 1, we have exchanged several amino acid residues of the *YPT* protein within the conserved segments likely to be involved in nucleotide binding and we subsequently analyzed the physiological and biochemical consequences of these mutations. The mutations were introduced into the gene with standard methods using synthetic oligonucleotides (Zoller and Smith, 1982; Pielak *et al.*, 1985), and they were verified by sequence analysis.

The wild-type *YPT1* gene on chromosome VI was then exchanged by the different mutant genes contained in a 6.9 kb
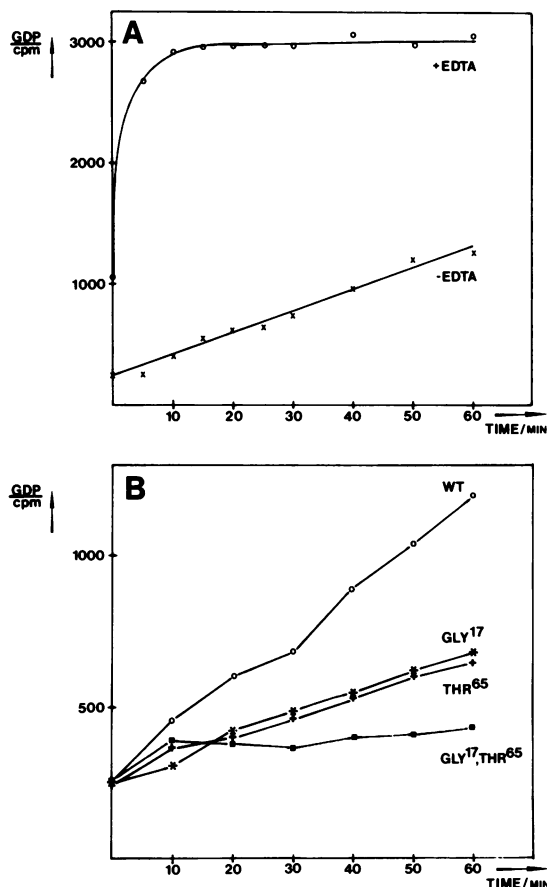
Fig. 3. GDP exchange reactions of *YPT* wild-type and mutant proteins. The reactions were performed at 20°C in a final volume of 1 ml with 0.12 μM active protein and 10 μM [^{3}H]GDP (500 c.p.m./pmol) in low Mg^{2+} (1 mM MgCl$_2$ and 2 mM EDTA) (**A**) or 10 mM MgCl$_2$ (**B**) In A, purified wild-type *YPT* protein was used for the nucleotide exchange reaction in low and high Mg^{2+}. At the time points indicated, 50 μl were filtered through nitrocellulose filters and the radioactivity was counted after washing the filters.

*Hind*III fragment which, in addition, carried the *LEU2* gene as selectable marker inserted into the *Bam*HI restriction site downstream of the *YPT1* gene (Schmitt *et al.*, 1986). Diploid transformants carrying the *YPT1* mutations at the proper site of one chromosome VI were subjected to spore analysis in order to search for mutant phenotypes.

In parallel, the wild-type and the mutant *YPT1* gene were expressed in *E. coli* and the relative GTP-binding affinities of the bacterially produced proteins were analyzed either on nitrocellulose filters to which equal amounts of the different proteins were bound (Figure 2) or by employing the nucleotide exchange reaction where the bound GDP was exchanged with [^{3}H]GDP in the presence of 10 mM Mg^{2+} at room temperature (Figure 3B).

The rationale behind the mutations introduced into the *YPT1* gene and the results obtained were as follows.

(i) The Lys^{21} → Met substitution was thought to disrupt a possible ionic contact between a phosphate group of the nucleotide and the protein. This seemed likely because of the conserved structure of Gly-x-x-x-x-Gly-Lys in several nucleotide binding proteins, including the adenylate kinase where this sequence motif forms a loop surrounding the phosphate moiety of AMP (Pai *et al.*, 1977). We observed that this mutation of the *YPT* protein was lethal and abolished or dramatically reduced the protein's

capacity to bind GTP when measured on nitrocellulose filters (Figure 2, lane 4).

(ii)Several GTP-binding proteins studied have the conserved sequence Asn-Lys-x-Asp that is preceded by a hydrophobic region (Figure 1). This conserved segment is identical in the human *ras* proteins and the yeast *YPT* protein. X-ray diffraction studies of the GTP-binding *E. coli* elongation factor EF-Tu have established that the conserved asparagine residue (in position 135) appears to make several contacts with the guanine ring of the bound GDP (La Cour *et al.*, 1985; Jurnak, 1985). As we reported recently, the Asn^{121} → Ile substitution in the *YPT* protein led to a dominant lethal phenotype. As can be seen in Figure 2, lane 7, the mutated protein synthesized in *E. coli* had no detectable capacity to bind GTP. In trying to purify the *YPT^{met21}* and the *YPT^{ile121}* mutant proteins expressed in *E. coli* we encountered the problem that the two proteins were very unstable and resisted a satisfying purification for meaningful nucleotide binding assays in solution.

(iii) The intrinsic GTPase activity of the mammalian *ras* proteins is decreased following changes of either the Gly^{12} or Ala^{59} residues (Gibbs *et al.*, 1984a; Manne *et al.*, 1984; Sweet *et al.*, 1984). Both amino acid residues are conserved in different GTP-binding proteins, with the exception of the viral *ras* gene products (where both residues are changed) and the *YPT* protein, which has a serine in position 17, corresponding to Ser^{12} in the viral K-*ras* protein (Figure 1). Since the other two yeast *ras* homologues, *RAS1* and *RAS2*, have a glycine in the corresponding position, we chose to study the effect of the Ser^{17} → Gly substitution in the *YPT* protein. This mutation led to perfectly viable cells. The GTP-binding capacity of the mutant protein, analyzed after its fixation to nitrocellulose, seemed to be reduced compared to that of the wild-type protein (Figure 2, lane 5). In keeping with this result, the capacity for GDP binding of the *YPT^{gly17}* mutant protein measured in solution was also reduced compared to the wild-type protein (see Figure 3B). As we will show below, the GTPase activity of this mutant protein was also changed.

(iv) Viral *ras* proteins contain a threonine residue in position 59 (Figure 1). This threonine replaces an alanine residue found in normal cellular *ras* gene products and it is the site of auto-phosphorylation of viral *ras* proteins (Shih *et al.*, 1982). The mutation of the chromosomal *YPT1* gene resulting in an Ala^{65} → Thr substitution (Figure 1), which corresponds to the Ala^{59} → Thr exchange in the viral *ras* protein, led to viable cells with unchanged growth properties. The GTP-binding assay with the mutant protein fixed to nitrocellulose filters suggested a reduced capacity to bind the guanine nucleotide as compared to wild-type protein (Figure 2, lane 6). Here again, with the nucleotide exchange reaction in solution, a decreased GDP-binding capacity of the mutant protein was observed (Figure 3B).

Severe effects of cellular growth were observed with the double mutant *YPT^{gly17,thr65}*. Spores expressing only the *YPT^{gly17,thr65}* mutant gene were able to germinate and gave rise to microcolonies with up to 40 to 60 cells within 3 days. At this time several cells of the microcolonies displayed the multibudded, 'Micky Mouse'-like phenotype that we observed in *YPT*-deficient cells after the shut-off of *YPT1* gene transcription (Schmitt *et al.*, 1986). It is noteworthy that the growth of haploid cells overexpressing the *YPT^{gly17,thr65}* mutant gene under the control of the inducible *GAL10* promoter did markedly improve.

The functional defect of the protein mutated in positions 17 and 65 is likely to be explained by its changed biochemical properties. As shown by the filter binding test using [^{32}P]GTP
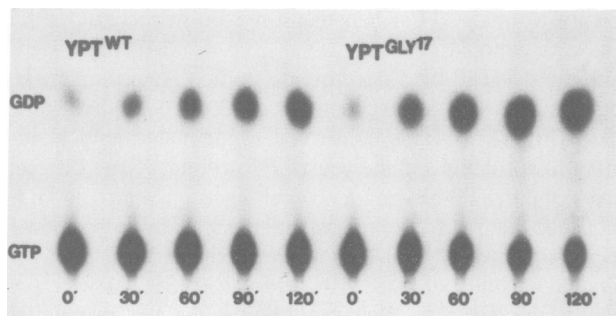
Fig. 4. GTP hydrolysis by wild-type *YPT* protein and mutant *YPT^gly17* protein. Bacterially produced, purified proteins (0.25 μM final concentration of active protein) were incubated at 30°C with [α-^{32}P]GTP (20 μM final concentration). Aliquots were withdrawn from the incubation mixture at the time points indicated and GTP and GDP were separated on PEI plates and visualized by autoradiography.

Fig. 5. Kinetics of GTP hydrolysis. GTP hydrolysis catalyzed by the different *YPT* proteins was followed as described in the legend to Figure 4. Spots corresponding to GDP were excised from the PEI plates and counted. Data presented are the mean of two independent measurements.

(Figure 2, lane 8) as well as by the GDP exchange reaction performed in solution (Figure 3B), the binding capacity for guanine nucleotides of the mutant protein was significantly reduced.

The YPT protein possesses an intrinsic GTPase activity

Guanine nucleotide binding proteins are usually activated by the reversible binding of GTP. The transient nature of this activation is believed to be conferred on these proteins by their intrinsic GTP hydrolytic activity (Gilman, 1984; Gibbs *et al.*, 1985).

Taking advantage of the availability of purified wild-type and mutant *YPT* proteins synthesized in *E. coli*, we undertook a study of the GTPase activity of the *YPT* protein and its possible impairment by the different mutations introduced into the protein. Equal amounts of active protein were incubated with [α-^{32}P]GTP, and GTP hydrolysis was followed by the generation of GDP for up to 2 h. Labelled GTP and GDP were separated by thin-layer chromatography on PEI-plates and the GDP formed was measured by Cerenkov counting. It should be mentioned that with this method the GDP released as well as the protein-bound GDP is measured.

As can be seen in Figure 4 and 5, the *YPT* protein possesses GTPase activity and this activity was altered to a varying degree in the different mutant proteins. The time course presented in Figure 5 indicates that, under the conditions employed, the GTPase activity was linear for at least 2 h for the wild-type and the mutant *YPT* proteins.

From the slopes of the curves the estimated rate of GTP hydrolysis was about 0.006 min^{-1} for the wild-type *YPT* protein, which is significantly lower than the rate of hydrolysis of 0.02 min^{-1} determined for the mammalian p21 *ras* gene products (Temeles *et al.*, 1985; Gibbs *et al.*, 1985). The Ser^{17} → Gly substitution increased the GTP hydrolytic activity by a factor of about 2.5 (0.015 min^{-1}). In contrast, the GTPase activity of the *YPT^thr65* mutant protein was reduced by a factor of about 4 (0.0015 min^{-1}) whereas that of the *YPT^gly17,thr65* protein was nearly the same as that of the wild-type protein's activity.

The Ala^65 → Thr substitution confers on the YPT protein autophosphorylating activity

The change of the alanine residue in position 65 of the *YPT* protein to a threonine residue, analogous to the Ala^{59} → Thr substitution found in viral *ras* proteins, not only impaired the GTPase activity of the mutant protein but also imposed on the yeast protein autophosphorylating activity. The *YPT* wild-type
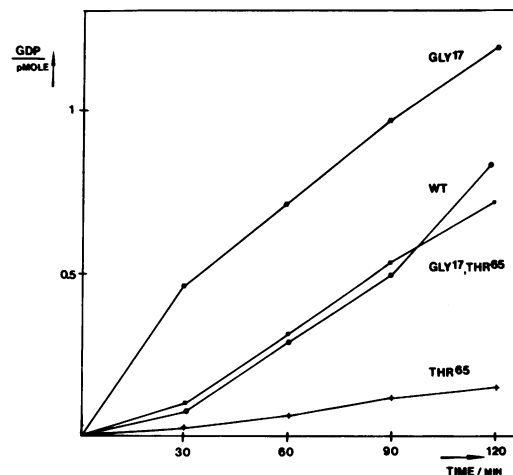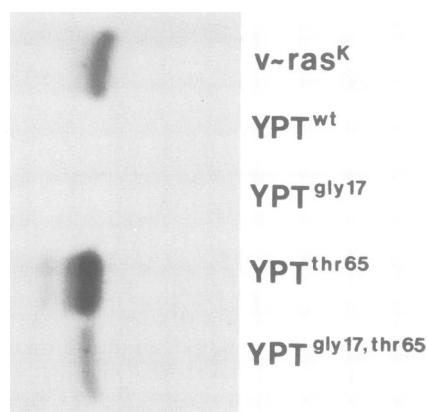

Fig. 6. Autophosphorylation of viral K-*ras* and *YPT* mutant proteins. Bacterially produced, purified protein (1 μM final concentration of active protein) was incubated with [γ-^{32}P]GTP (20 μM final concentration) at 30°C for 1 h. One-third of the incubation mixture was treated with SDS-containing buffer, boiled, and subjected to SDS–PAGE using a 12.5% polyacrylamide gel. The gel was dried and autoradiographed.

protein and different mutant proteins as well as the viral K-*ras* protein were synthesized in *E. coli* and the purified proteins were incubated with [γ-^{32}P]GTP for up to 3 h at 30°C. Aliquots of the protein solutions were then mixed with SDS-containing buffer, heated, and subjected to SDS–PAGE.

As can be seen from the autoradiogram of the dried gel (Figure 6), the *YPT* mutant protein with the Ala → Thr substitution in position 65, but neither the *YPT* wild-type nor the *YPT^gly17* mutant protein, were competent for autophosphorylation. As a control, the viral K-*ras* protein with the analogous Ala^{59} → Thr substitution (see Figure 1) was included in this analysis and can be seen to exhibit its known capacity for autophosphorylation. It is also evident from Figure 6 that the additional exchange of serine-17 to glycine significantly reduced the autophosphorylating activity of the *YPT^thr65* mutant protein. From a kinetic analysis shown in Figure 7, the rates of autophosphorylation were estimated to be 0.0011 min^{-1} and 0.0005 min^{-1} for the *YPT^thr65* and the *YPT^gly17,thr65* protein, respectively.
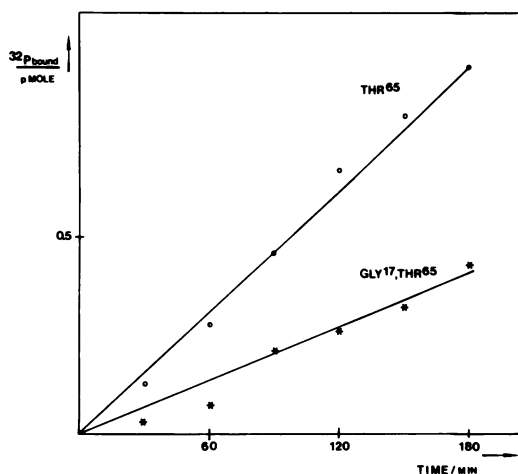
Fig. 7. Kinetics of autophosphorylation of YPT^{thr65} and $YPT^{gly17,thr65}$ mutant proteins. Experimental conditions were as described in Materials and methods.

Discussion

The main conclusion that can be drawn from the mutational analysis of the *YPT* protein is that the biochemical properties of this *ras*-like yeast protein and the mammalian p21 *ras* gene products are very similar. The *YPT* protein binds GTP specifically, it is able to hydrolyze GTP and the mutant form of the protein with an Ala → Thr substitution within one of the highly conserved protein regions possesses autophosphorylating activity. Moreover, the mutations that we have introduced into the *YPT* protein within the regions sharing identical primary structure with the mammalian *ras* proteins, resulted in characteristic alterations of the biochemical properties of the yeast protein that have also been observed in comparable mammalian mutant *ras* proteins. Some of the *YPT* mutations gave rise to rather distinct lesions of cellular growth.

Biochemical properties of the YPT protein

Molecular models of two GTP/GDP-binding proteins, the *E. coli* elongation factor EF-Tu and the human p21 *ras* protein, have recently been proposed (Jurnak, 1985; La Cour *et al.*, 1985; McCormick *et al.* 1985). The *ras* protein tertiary structure model was constructed from X-ray crystallographic information of the GDP-binding domain of EF-Tu and the known conserved sequence motifs in several guanine nucleotide-binding proteins, and it was supposed that the GDP-binding domain of EF-Tu was structurally similar to that of the p21 *ras* protein (McCormick *et al.*, 1985).

According to these models two loops, connecting a β-strand and an α-helix, are involved in contact formations with the phosphate groups (see Figure 1A,B), and another loop, Asn-Lys-Cys-Asp (see Figure 1C), interacts with the guanine ring of the nucleotide. These sequence motifs are also conserved in the *YPT* protein, and two of the four mutations we have introduced into these regions of the *YPT* protein clearly affected the binding of guanine nucleotides.

In designing the mutations we only considered substitutions that would primarily change the charge of the residue in question. Lys[21] of *YPT*, which corresponds to lysine in position 24 of EF-Tu or Lys[16] of vertebrate *ras* proteins, was thought to form ionic contacts with a phosphate group of the nucleotide. Charge neutralization of one phosphate group of the bound GDP by the side chain amino group of Lys[24] of EF-Tu appears to be

likely (Jurnak, 1985). It was pleasing, therefore, to find the Lys[21] → Met substitution of the *YPT* protein to reduce severely or abolish the binding of GTP to the protein, a result consistent with the assumptions made. Our finding is in accord with a recent report of Sigal *et al.* (1986) who observed a 100-fold reduction in the affinity for GTP and GDP of an H-*ras* mutant protein with a Lys[16] → Asn substitution.

The importance of the glycine residue in position 12 of the human *ras* proteins for the proper functioning of these proteins has been shown in many studies. Substitution of this residue by other amino acids, except proline, was found to confer transforming activity on the *ras* proteins (Seeburg *et al.*, 1984). It has been discussed that the biochemical basis for the observed transforming activity might be a decrease in the p21-associated GTPase activity (McGrath *et al.*, 1984; Gibbs *et al.*, 1984a; Sweet *et al.*, 1984; Manne *et al.*, 1985; Temeles *et al.*, 1985; Colby *et al.*, 1986), but this is still a matter of controversy. The GTP-binding affinity, however, was found to be unchanged between the normal and the transforming p21 *ras* protein mutated in codon 12 (McGrath *et al.*, 1984; Gibbs *et al.*, 1984a). The shortcoming of these studies, namely the use of nucleotide-bound p21 *ras* proteins, has recently been overcome by the investigation of Feuerstein *et al.* (1987) who used nucleotide-free *ras* proteins to determine the binding constants from measurements of the on and off rates of GDP in the presence or absence of Mg^{2+} ions. According to their calculations the binding constants of normal and mutant p21 *ras* proteins are in the range of $10^9 - 10^{11}$ M^{-1}, the binding constant of the transforming H-*ras* protein with a Gly → Val substitution in position 12 being higher by only a factor of about 2.

The 2-fold higher relative GDP-binding capacity of the *YPT* wild-type protein with a serine residue in position 17 compared to the YPT^{gly17} mutant protein that we observed by following the kinetics of the GDP exchange reaction is consistent with the slight difference in the binding constants measured for wild-type and transforming *ras* proteins (Feuerstein *et al.*, 1987). It is clear, however, that our investigation does not allow distinction between on and off rates and that a comparison of the *YPT* and *ras* proteins with respect to their nucleotide-binding properties has to be performed with nucleotide-free protein. We would like to stress that the decrease of the relative GDP-binding capacity of the different *YPT* mutant proteins in solution was likewise observed with the proteins fixed to nitrocellulose filters after SDS−PAGE.

The usefulness of the filter assay for measuring the apparent nucleotide affinity of *ras* proteins has already been documented by others (McGrath *et al.*, 1984). The advantage of this method is that the nucleotides bound to the isolated proteins are stripped off the protein during its denaturation in SDS-containing buffer and the electrophoresis in SDS−polyacrylamide gels. There exists, however, the possibility that the various mutant proteins differ in their renaturation behaviour during the time of the removal of SDS in the gels, the electrophoretic transfer to nitrocellulose filters, and on the filters themselves. As the relative guanine nucleotide-binding affinities of the wild-type and mutant *YPT* proteins that we observed both by GTP binding to nitrocellulose-fixed proteins and by following the kinetics of GDP exchange of the purified proteins in solution were similarly different, we consider the filter binding assay to be meaningful without major reservations. Although two of the mutant proteins (Lys[21] → Met and Asn[121] → Ile substitutions) could not be studied in solution so far, we believe that the inability to detect any GTP-binding activity by the filter binding assay reflects the complete loss or

a drastic decrease of the affinity of these mutant proteins for guanine nucleotides. This is supported by our finding that the two mutations rendered the proteins unfunctional and caused lethality.

As pointed out above, the Asn^{121} → Ile substitution concerns a residue of the loop structure Asn−Lys−Cys−Asp that is conserved in the yeast *YPT* protein, the mammalian *ras* proteins and the *E. coli* elongation factor EF-Tu and which, according to X-ray crystallographic studies of the EF-Tu protein, participates in the interaction with the guanine ring of the bound nucleotide. It appears that the asparagine residue forms several contacts and that the aspartic acid of the loop interacts with the amino group of the guanine ring (La Cour *et al.*, 1985; Jurnak, 1985). The satisfying experimental proof for the importance of the conserved asparagine residue in GTP binding comes from the mutation Asn^{121} → Ile, which we have performed in this study and which was found to render the *YPT* protein defective in GTP binding. Substitution of the corresponding asparagine residue in the mammalian *ras* gene product with either lysine or tyrosine likewise resulted in the loss of GTP binding (Clanton *et al.*, 1986).

Similar to the vertebrate *ras* proteins, the Ala^{59} → Thr substitution of which results in an autophosphorylation with the threonine being the phosphate acceptor site (Shih *et al.*, 1980), the Ala^{65} → Thr exchange of the *YPT* protein also led to an autophosphorylation of the protein. The model for the tertiary structure of the p21 *ras* protein suggests a close proximity of the γ-phosphate group of the bound GTP to Ala^{59} (McCormick *et al.*, 1985), and because of the comparable biochemical properties of the *ras* and the *YPT* proteins, this might be true also for the yeast protein.

As with the mammalian *ras* proteins, the GTPase activity of the *YPT* protein was found to be elevated in a mutant protein with a glycine residue in position 17 instead of the serine residue present in the wild-type protein. In addition, the Ala→Thr substitution in position 65 of the *YPT* protein led to a decrease in the GTP hydrolytic activity as is the case for the same mutation in mammalian *ras* proteins (Temeles *et al.*, 1985). Furthermore, the observed decrease in the autophosphorylating activity of the mutant *YPT*^{gly17,thr65} protein compared to the *YPT*^{thr65} protein having a serine residue in position 17 is reminiscent of the observation with mammalian *ras* proteins, where a change in residue 12 (corresponding to residue 17 in the *YPT* protein) modulates the extent of autophosphorylation (Gibbs *et al.*, 1984b). These similarities again stress the validity of our postulation that the high degree of sequence homology between the yeast *YPT* and the mammalian *ras* proteins implies very similar tertiary structure of these proteins (Gallwitz *et al.*, 1983).

Effect of mutant YPT proteins on cell viability

Although all of the *YPT* mutant proteins analyzed had altered biochemical properties, different effects were observed with regard to the viability of haploid cells that expressed one of the mutant genes which replaced the wild-type *YPT1* gene on chromosome VI. Lethality in two cases (Lys^{21} → Met and Asn^{121} → Ile substitution) most likely reflects the inability or a severe impairment of the mutant proteins to bind GTP. The dominant-lethal phenotype of cells expressing the *YPT*^{ile121} mutant protein could be envisaged as the result of a 'locked in' conformation of this protein, leading to a tight or irreversible complex with an effector protein and subsequently to a mitotic lesion (Schmitt *et al.*, 1986).

The double mutant *YPT*^{gly17,thr65} with a significantly reduced GTP-binding ability gave rise to ill-growing cells, several of them

with a phenotype resembling that of cells depleted of *YPT* protein (Schmitt *et al.*, 1986). It is interesting to note that cells overexpressing this mutant protein regained nearly normal growth properties. This could mean that an increase of the intracellular level of this mutant protein suffices to retain normal cellular growth by compensating either for its reduced nucleotide-binding capacity or the mutant protein's reduced stability. So far we have not investigated these possibilities.

On the other hand, cells expressing the *YPT* mutant proteins with moderately decreased GTP-binding capacity (*YPT*^{gly17}, *YPT*^{thr65}) had virtually unchanged growth properties. The simultaneous increase (*YPT*^{gly17}) or drop (*YPT*^{thr65}) in the GTP hydrolytic activity of the two mutant proteins, therefore, did not detectably alter the generation time of the cells measured in rich growth medium. These findings show that subtle changes in the GTP-binding capacity and/or the GTPase activity of the essential *ras*-like *YPT* protein is not harmful to the cells, at least under favourable growth conditions. This conclusion bears also on the controversially discussed matter whether altered GTPase activity of mutant *ras* protein is causally related to their acquirement of transforming activity (McGrath *et al.*, 1984; Gibbs *et al.*, 1984a; Sweet *et al.*, 1984; Manne *et al.*, 1985; Temeles *et al.*, 1985; Der *et al.*, 1986; Lacal *et al.*, 1986).

Materials and methods

Cloning and oligonucleotide-directed mutagenesis

Cloning in bacterial plasmids and phage M13 was employed and *in vitro* mutagenesis was performed as described (Schmidt *et al.*, 1986). The following oligonucleotides were used: 5′-TCCCGACACCGCCATTCCCGATCA-3′ to mutate codon 17; 5′-AACAGGACATCCCGACACCG-3′ to mutate codon 21; 5′-TCTTGACCTGTAGTGTCCCAA-3′ to mutate codon 65, and 5′-CACACTTGATCCCTACCA-3′ to mutate codon 121. Mutants were checked either by sequencing or with the ^{32}P-endlabelled primer.

Expression of wild-type and mutant YPT proteins in bacteria

For the bacterial expression of *YPT* proteins, a pUC8-derived expression vector, pPLN, was constructed with a single *Nde*I restriction site at the start of the *lacZ* gene. An *Nde*I restriction site was also created by oligonucleotide-directed mutagenesis at the start of the *YPT1* gene which allowed the insertion of the *YPT1* gene or its mutated versions into the expression vector as an *Nde*I/*Bam*HI fragment. *E. coli* strains RR1 or JM101 were transformed with these vectors and the yeast proteins were expressed by induction with 1 mM IPTG for 3 h. One ml of the bacterial suspension was centrifuged, the bacterial pellet was washed in 1 ml of 50 mM Tris−Cl, pH 7.5, 0.15 M NaCl and taken up in 200 μl of an SDS-containing buffer according to Laemmli (1970). The extract was boiled for 5 min and 25 μl were subjected to SDS−PAGE.

Purification of bacterially produced YPT proteins

Cells in early log phase were induced for 3 h with 0.5 mM IPTG. Bacteria from a 6 l culture (about 15 g wet weight) were pelleted, washed with 50 mM Tris−Cl, pH 8, 0.1 M NaCl, suspended in 50 ml of buffer A (20 mM Tris−Cl, pH 8, 1 mM sodium azide, 10 mM MgCl$_2$, 1 mM PMSF), and disrupted by sonication for 3 min with six 30-s intervals on ice. After precipitating cellular debris and unbroken cells by centrifugation, the supernatant was diluted to 100 ml with buffer A and loaded onto a 500 ml DEAE−Sephacel column. Proteins bound to the ion exchanger were eluted with a 1200 ml 0−400 mM NaCl gradient Aliquots of the column fractions were spotted onto nitrocellulose filters to identify the *YPT* protein by either exploiting its GTP-binding capacity or by using antibodies prepared against the wild-type *YPT* protein. *YPT* proteins eluted at about 0.12 M NaCl, relevant fractions were pooled, and the proteins were concentrated to 10 ml by ultrafiltration using an Amicon YM10 membrane. Further separation of the proteins was performed on a 120 cm × 2.5 cm column of Sephadex G-150 and, on average, 35−40 mg of *YPT* protein with a purity exceeding 90% was usually obtained. The purified proteins were stored in aliquots at −80°C.

YPT antibodies and immunoblot analysis

Antibodies against bacterially produced *YPT* protein were raised in rabbits. The purified protein was subjected to SDS−PAGE, the stained band was excised from the gel, crushed, and mixed with complete Freund's adjuvant. The immunization scheme was the same as described for the generation of antibodies against

a *YPT/MS2* polymerase fusion protein (Schmitt *et al.*, 1986). For immunoblot analysis, gels were treated and the separated proteins were transferred to nitrocellulose filters as described (Schmitt *et al.*, 1986). Protein blots of bacterially produced *YPT* proteins were stained with peroxidase-conjugated protein A (BioRad) according to the supplier's recommendations and using *YPT* protein antibodies.

Nucleotide binding

Nucleotide binding using [α-^{32}P]GTP was analyzed with the proteins separated by SDS−PAGE and transferred electrophoretically to nitrocellulose filters (Schmitt *et al.*, 1986).

The GDP exchange reaction was performed at 20°C in a final volume of 1 ml consisting of 20 mM Tris−Cl, pH 8, 5 mM DTT, 1 mM NaN$_3$ 10 μM [^{3}H]GDP (500 c.p.m./pmol), 10 mM MgCl$_2$ and 0.12 μM of YPT protein. To follow the nucleotide exchange under conditions of low Mg^{2+}, the solution contained 1 mM MgCl$_2$ and 2 mM EDTA instead of 10 mM MgCl$_2$. Fifty μl aliquots were taken at different times of incubation and filtered through nitrocellulose BA85, 0.45 μm (Schleicher and Schuell). The filters were washed twice with 3 ml of ice cold buffer (20 mM Tris−Cl, pH 8, 5 mM MgCl$_2$, 10 mM NH$_4$Cl, 1 mM 2-mercaptoethanol, 0.1 M KCl) and dried for 10 min at 80°C. Dried filters were dissolved in Filter-solv (NEN) and the radioactivity was measured by liquid scintillation counting.

GTPase and autophosphorylating activities

GTPase activity was determined at 30°C in a final volume of 200 μl consisting of 0.065 mM Tris−Cl, pH 7.6, 10 mM MgCl$_2$, 0.5 mM DTT, 1 mM NaN$_3$, 20 μM [α-^{32}P]GTP (8000 c.p.m./pmol), 1 mM ATP, 0.25 μM of active protein. ATP was included in the reaction to inhibit a contaminating phosphatase activity that was noted in some protein preparations. The ATP interfered neither with the GTPase nor with the autophosphorylation activity. At the time points indicated in the figures, duplicate samples of 10 μl were withdrawn from the incubation mixture, mixed with 10 μl ice cold 0.5 M EDTA and immediately frozen at −80°C. Two μl were spotted onto PEI plates, the plates were developed in 1 M HCOOH/1 M LiCl, dried and autoradiographed. The GDP spots were excised and the radioactivity was measured by Cerenkov counting.

Autophosphorylating activity was analyzed with 2.5 μg of the purified *YPT* wild-type or mutant proteins in 100 μl of the same buffer used for determining GTPase activity and 30 μCi of [γ-^{32}]GTP (spec. act. 17 Ci/mmole). Incubations were performed at 30°C for 60 min, 35 μl of the incubation mixture were mixed with an equal volume of SDS-containing buffer, boiled, and subjected to SDS−PAGE. The gels were dried and autoradiographed.

To follow the kinetics of autophosphorylation, the *YPT*^{thr65} and the *YPT*^{gly17,thr65} mutant protein (0.65 μM of active protein) were incubated at 30°C in a final volume of 300 μl with 20 μM of [γ-^{32}P]GTP (2500 c.p.m/pmol) in the buffer described above. At different time intervals 10 μl were filtered through nitrocellulose, the filters were washed four times with 2 ml each of ice cold 10% TCA, dried and the radioactivity was measured by Cerenkov counting.

Other methods

Preparation of yeast DNA, yeast transformation, and Southern analyses were as described previously (Langford and Gallwitz, 1983). Successful gene replacement was checked by Southern analysis using ^{32}P-nick translated *YPT1* gene fragments and ^{32}P-endlabelled oligonucleotides used to generate the mutant genes. In some cases the mutant *YPT1* genes were isolated from transformed yeast cells and sequenced according to Maxam and Gilbert (1980). Procedures for growth of yeast cells, sporulation, micromanipulation, and scoring of genetic markers were carried out by standard methods (Mortimer and Hawthorne, 1969).

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