The EMBO Journal vol.9 no.6 pp.1957 - 1962, 1990

Structural and functional analysis of *ypt2*, an essential *ras*-related gene in the fission yeast *Schizosaccharomyces pombe* encoding a Sec4 protein homologue

Heinz Haubruck[1], Ulrike Engelke, Peter Mertins and Dieter Gallwitz

Max-Planck-Institute for Biophysical Chemistry, Department of Molecular Genetics, PO Box 2841, D-3400 Göttingen, FRG

[1]Present address: Department of Molecular Biology, Cetus Corporation, Emeryville, CA 94608, USA

Communicated by D.Gallwitz

Using the cloned *Saccharomyces cerevisiae YPT1* gene as hybridization probe, a gene, designated *ypt2*, was isolated from the fission yeast *Schizosaccharomyces pombe* and found to encode a 200 amino acid long protein most closely related to the ypt branch of the ras superfamily. Disruption of the *ypt2* gene is lethal. The bacterially produced *ypt2* gene product is shown to bind GTP. A region of the ypt2 protein corresponding to but different from the 'effector region' of ras proteins is also different from that of ypt1 proteins of different species but identical to the 'effector loop' of the *S.cerevisiae SEC4* gene product, a protein known to be required for vesicular protein transport. The *S.pombe ypt2* gene under control of the *S.cerevisiae GAL10* promoter is able to suppress the temperature-sensitive phenotype of a *S. cerevisiae sec4* mutant, indicating a functional similarity of these GTP-binding proteins from the two very distantly related yeasts.

Key words: GTP-binding protein/ras superfamily/*Schizosaccharomyces pombe*/Sec4p homologue/*ypt2* gene

Introduction

Ras and ras-related proteins constitute a superfamily of eukaryotic proteins which, by a cycle of GDP and GTP binding, are thought to act as regulators in diverse cellular processes (for reviews see Barbacid, 1987; Chardin, 1988; Gallwitz *et al.*, 1989). All of these proteins are on average 200 amino acid residues long, and segments of these proteins, known from X-ray crystallographic analysis of the H-ras protein to interact with the bound guanine nucleotide (Pai *et al.*, 1989), are highly conserved. According to other structural features, at least three families among the superfamily of ras proteins can be distinguished: ras, rho and ypt proteins (Haubruck *et al.*, 1987; Gallwitz *et al.*, 1989).

Except for the Ras proteins in baker's yeast *Saccharomyces cerevisiae*, whose primary function is to regulate adenylyl cyclase activity (Toda *et al.*, 1985), the cellular function of neither of the multiple ras or ras-like proteins has so far been precisely defined. However, recent genetic, biochemical and cell physiological analyses with baker's yeast lend strong support to the assumption that members of the ypt family regulate the vectorial transport of vesicular structures in the pathways of protein transport (Goud *et al.*, 1988; Schmitt *et al.*, 1988; Segev *et al.*, 1988; Walworth *et al.*, 1989; Baker *et al.*, 1990).

In mammalian cells, the number of ypt proteins (also designated rab) seems to be rather large (Zahraoui *et al.*, 1989). In contrast, only two members of this protein family, the Ypt1 protein (Gallwitz *et al.*, 1983) and the Sec4 protein (Salminen and Novick, 1987) have been identified in the yeast *S.cerevisiae*. Both proteins are essential for cell viability (Schmitt *et al.*, 1986; Salminen and Novick, 1987). Whereas conditional-lethal *ypt1* mutants at the non-permissive temperature accumulate endoplasmic reticulum and are defective in protein secretion and calcium regulation (Schmitt *et al.*, 1988; Segev *et al.*, 1988), the phenotype of *sec4* mutants is characterized by a massive accumulation of small vesicles unable to fuse with the plasma membrane (Goud *et al.*, 1988; Walworth *et al.*, 1989). Whereas the Ypt1 protein is attached primarily to Golgi membranes (Segev *et al.*, 1988) via a lipid moiety covalently bound to a C-terminal cysteine (Molenaar *et al.*, 1988), the Sec4 protein localizes to post-Golgi vesicles and the plasma membrane (Walworth *et al.*, 1989). Recent cell-free protein transport studies indicate that Ypt1p is an essential component for the vesicular transport of proteins from the endoplasmic reticulum to the Golgi complex (Baker *et al.*, 1990).

As the mouse ypt1 protein (Haubruck *et al.*, 1987), also designated rab1 (Touchot *et al.*, 1987), is able functionally to replace the *YPT1* gene product in *S.cerevisiae* (Haubruck *et al.*, 1989) and is highly enriched in Golgi structures (Segev *et al.*, 1988; M.Puzicha, G.Dressler, H.Haubruck and D. Gallwitz, unpublished), it seems likely that this protein fulfils a similar function also in mammalian cells. However, among the different ypt (rab) proteins identified in mammalian species, no Sec4 homologue has so far been identified.

The fission yeast *Schizosaccharomyces pombe*, only distantly related to *S.cerevisiae* but also amenable to easy genetic analyses, is thought to be a better model organism in comparative studies with higher eukaryotes (Russel and Nurse, 1986). As the ras proteins in the two yeasts are integrated into different regulatory pathways (Toda *et al.*, 1985; Fukui *et al.*, 1986), it is expected that a complementary study on the function of other GTP-binding regulatory proteins will be extremely instructive. We here report the isolation of a gene, *ypt2*, from *S.pombe* whose product is shown to be a GTP-binding protein functionally identical to the *S.cerevisiae* Sec4 protein. The *ypt2* gene, like the *ypt1* and the *ypt3* gene described in the accompanying paper (Miyake and Yamamoto, 1990), encodes an essential function, a finding which indicates that the different members of the *ypt* gene family have vital and very discrete regulatory functions.

Results

Cloning, sequence and transcripts of the ypt2 gene

By Southern blot analysis with *Hin*dIII-digested total *S. pombe* DNA and a ^{32}P-labelled 0.53 kb *Eco*RI–*Hin*cII

fragment of the *S.cerevisiae YPT1* protein-coding region as hybridization probe, three hybridizing DNA fragments of ~9, 3 and 0.5 kb could be identified under moderately strong hybridization conditions (6 × SSC, 60°C). A 9.2 kb *Hin*dIII fragment obtained by shotgun cloning was subjected to restriction endonuclease mapping, and a 2.65 kb *Nco*I fragment found to react with the *S.cerevisiae YPT1* hybridization probe (see Figure 5) was cloned into the *Hin*cII restriction site of plasmid pUC8 and partially sequenced.

An open reading frame of 200 codons was detected (Figure 1) the amino acid sequence deduced showed clear resemblance to the Ypt1 protein sequence of baker's yeast. The gene, first designated *ypt1*, was renamed *ypt2* after the recent identification in *S.pombe* of the *S.cerevisiae YPT1* gene homologue (Fawell *et al.*, 1989; Miyake and Yamamoto, 1990).

To identify transcripts of the putative gene, RNA blot analysis was performed using as hybridization probes either a 0.5 kb *Acc*I/*Sma*I fragment of the protein-coding region (Figure 5) or a 0.95 kb *Hin*cII fragment representing the last 12 codons and 920 bp of 3′ non-translated sequence. With either hybridization probe, three transcripts of ~1350, 1550 and 1800 nt were observed (Figure 2).

The ypt2 gene product is a GTP-binding protein

According to DNA sequence analysis, the *S.pombe ypt2* gene encodes a protein of 200 amino acid residues with a molecular mass of 22 757 daltons. Certain structural features regarded to be diagnostic for ypt proteins, a serine instead of glycine found in position 12 of mammalian ras proteins and two consecutive cysteine residues at the carboxyl-terminal end, for instance (Haubruck *et al.*, 1987; Gallwitz *et al.*, 1989), are also found in the ypt2 protein. Within the first 170 amino acid residues, the most highly conserved region of ras and ras-related proteins (Gallwitz *et al.*, 1983, 1989; Barbacid, 1987), the ypt2 protein exhibits identities to a degree of 59.3 and 64.7% with the *S.cerevisiae* Ypt1 and Sec4 protein respectively, and 76.6% with the *Dictyostelium discoideum sas1* gene product (Figure 3). By comparison, the degree of homology in this region with ras proteins from different eukaryotic species ranges between 36 and 39%.

In particular, the *S.pombe* ypt2 protein contains the three sequence elements $GX_4GK^S/_T$ (amino acids 16–22), DX_2G (amino acids 64–67) and NKXD (amino acids 122–125) noted to be highly conserved in different families of GTP-binding proteins (Dever *et al.*, 1987). These sequences, in addition to a fourth region conserved in ras and ras-like proteins, EXSA (amino acids 150–153 in ypt2p), have recently been shown by X-ray crystallographic analysis of the GTP-bound form of the c-H-ras protein to participate in nucleotide binding (De Vos *et al.*, 1988; Pai *et al.*, 1989).

To verify that the *S.pombe* ypt2 protein binds guanine nucleotides, the protein-coding region was inserted into the previously described pUC8-derived vector pLN (Wagner *et al.*, 1987) and expressed in *Escherichia coli*. The bacterially produced protein was then subjected to a GTP-blot analysis and found to bind [α-^{32}P]GTP, although with an apparently lower capacity than the *S.cerevisiae* Ypt1 protein (Figure 4).

The ypt2 protein is essential for cell viability

The existence in *S.pombe* of several genes belonging to the ras superfamily poses the question as to their functional

```
          -330                                  -300
5'-GCTAGTAAATTGTTTAGTGACAGAGATTTTTCTCTGACATGAAATCTGAATTATTTC
          -270                                  -240
TCTTGTGAATATCTTTGTTACTTTCTCAAGAGTGTATGTATGTGCCCTAGAATAGTGACT
          -210                                  -180
TCCTTTATCAAAACTTCGAGTTTATTGCCCTTAGGAAACGTACATAAAAAAGTTGAAAGT
          -150                                  -120
CATGTTTTTTCAACATAAAAAAACACAAACAAAGACTTAAATAAAACTAGTACCATTTAT
           -90                                   -60
ACAATAACATTACTTGAATTAATAAATTTAAGTGTATTTTTGCGGCATTATCTCAAGGGA
           -30                                    MetSerThrLys
CTCCAGTAGATTCTACCACTTACCACCTGGCAAAAGCAACACTCCTCCATGTCTACAAAA
            10                                    20
SerTyrAspTyrLeuIleLysLeuLeuLeuIleGlyAspSerGlyValGlyLysSerCys
TCCTACGATTATTTAATCAAATTACTTCTCATAGGAGATTCAGGTGTGGGAAAATCATGT
            30                                    40
LeuLeuLeuArgPheSerGluAspSerPheThrProSerPheIleThrThrIleGlyIle
CTTCTTCTGAGATTTTCGGAAGACTCGTTTACCCCTTCTTTTATCACAACGATTGGAATT
            50                                    60
AspPheLysIleArgThrIleGluLeuAspGlyLysArgIleLysLeuGlnIleTrpAsp
GATTTCAAGATTCGCACGATTGAACTAGATGGAAAACGTATCAAACTCCAAATTTGGGAC
            70                                    80
ThrAlaGlyGlnGluArgPheArgThrIleThrThrAlaTyrTyrArgGlyAlaMetGly
ACTGCTGGCCAGGAACGTTTCCGTACCATTACCACTGCTTATTATCGTGGTGCAATGGGT
            90                                   100
IleLeuLeuLeuTyrAspValThrAspLysLysSerPheAspAsnValArgThrTrpPhe
ATTTTATTGTTATACGATGTAACTGATAAAAAATCATTTGATAACGTCCGAACTTGGTTT
           110                                   120
SerAsnValGluGlnHisAlaSerGluAsnValTyrLysIleLeuIleGlyAsnLysCys
AGTAACGTTGAACAACATGCTAGTGAAAATGTTTATAAAATCCTTATTGGAAATAAATGT
           130                                   140
AspCysGluAspGlnArgGlnValSerPheGluGlnGlyGlnAlaLeuAlaAspGluLeu
GATTGTGAGGATCAGCGTCAGGTCTCCTTTGAACAGGGACAAGCGTTAGCCGATGAGCTG
           150                                   160
GlyValLysPheLeuGluAlaSerAlaLysThrAsnValAsnValAspGluAlaPhePhe
GGCGTTAAATTTTTAGAAGCTAGTGCGAAGACGAACGTCAATGTAGATGAGGCATTTTTT
           170                                   180
ThrLeuAlaArgGluIleLysLysGlnLysIleAspAlaGluAsnGluPheSerAsnGln
ACCCTTGCCCGGGAAATAAAAAAACAAAAGATTGATGCAGAAAACGAATTTTCCAACCAG
           190                                   200
AlaAsnAsnValAspLeuGlyAsnAspArgThrValLysArgCysCysStp
GCAAATAACGTTGACCTCGGAAACGATCGTACGGTGAAGAGGTGTTGTTAGGCTCCTTAT
          +20                                   +50
TATTATCTTGTCACATTTTCTTTTTCGCTAGTTTTTGATATTAGCACCGCCGATACGTAT
          +80                                  +110
ATAGTCGTGAAGTGTGTGGAGAGCTTGAAGGAGTGGAGATTGGCATTGCTTTTTATATCT
         +140                                  +170
TGTGTTGGTTATCGTTTCCGGATTGCTACTTTGTTTCCTTACTCTTTTCAACTCTACCCG
         +200                                  +230
GTTGGGAAGTTTATAATATTTCTCTATATTGTACACTTTCTCTTACTTTTTAATGCTTTC
         +260                                  +290
ATAACGTAAAATTTATGCCTAATAGCTTCAATCAACTTTTGATATGCTTTCGAGTTGTTG
         +320                                  +350
TGATTAGATCGAGTTTACATTTTGGAATATAATCAATTCCCATGTTTTTTTTTTCAACGT
         +380                                  +410
CTCTTTTTTCATTGCAATTTCCTTAACATTTCAAAAAGTAATGCCTTTGCTTTTTCTTGG
         +440                                  +470
GGCCATTTTCCAATGTTTCTCGCGAATCCTTTTATCAATTTGCAACGAGCAATCAAGCAC
         +500                                  +530
CATCTAATTCGTCCTGTTGATCTACCTCATTTATTTTTTACGGCTGCTGCCTATTGTTTT
         +560                                  +590
AAATGTTTGGGCCACTGAACCAAGTTTACATTTTTCATCATGTTATACCGATTTGATTAG
         +620                                  +650
GTTGTCATCAATCGAAACGTCTTTTAACGCCTATCTTTTACATATTTTAATTGCCTTATC
         +680                                  +710
TTCATCTTCATTTAAACGTAAGATGACTACTTACCTTGTTACATTGTAGATTTTATTTTA
         +740                                  +770
TTTTTATCTTGCAACGAGACCACAACAGAAGCCTTTCCAAGATTACCACTTCAGCATCTT
         +800                                  +830
AATCTTTTTAAACAACGTATTACTATATGAAAGTCGATGTACCAGATTATTGCCAAGATT
         +860                                  +890
AAAACGATTTCAGCAATGTAGAGTAATTTGGCATACTCATACTCTTGTTTGTTTGCGTTT
         +920                                  +950
TTGTTAACTACGCAATTGCTAACCAAGATAACGATGTGTTACTTTGCGGTAAGTCTCAAC
         +980                                 +1010
TTGGGATTTTACTATTTTCTTTTCATGACAATTTAACGATATGCAATTATATATATATCG
        +1040                                 +1070
ATAGTCAGGTTGGACTTAATGTACAATTACCAGCTGATAGCTGATAATTGAAATATGAAA
        +1100                                 +1130
CGCAGGAAATTTGATTCAATTTATAATATTTTCATTTTAGGATATATTAAAAATTACGAA
        +1160                                 +1190
TACATTTCCGTTTTGGGTGACAAATATAAAACTTAAGAACAAAAAATGTAGCGAAG-3'
```

Fig. 1. Nucleotide and deduced amino acid sequence of the *ypt2* gene. Nucleotides upstream of the initiation codon are numbered negatively, those downstream of the stop codon (stp) are numbered positively.

interchangeability. As it is fairly easy to inactivate precisely a given chromosomal gene in yeast and to enquire into the dependence for cell viability of its protein product, the *ypt2* gene on one chromosome was disrupted in the *ura4*$^-$ diploid strain UL130. This was achieved by transforming cells with a linear 4.75 kb *Hin*dIII–*Pst*I fragment which has the 1.6 kb *Acc*I/*Cla*I fragment (*ypt2* codons 3–200 and ~1000 nt of 3′-untranslated region) replaced by the *S. pombe ura4* gene on a 1.8 kb *Cla*I fragment (Figure 5). The Southern blot analysis of three *ura4*$^+$ transformants, showing correct chromosomal integration of the *ypt2*-disrupting fragment, is presented in Figure 5. After

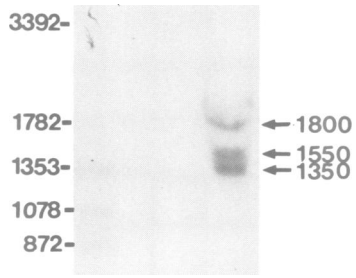

Fig. 2. RNA blot analysis to identify *ypt2* gene transcripts. Total cellular RNA was glyoxylated, separated on a 1% agarose gel and transferred to nitrocellulose. Hybridization was performed with a ^{32}P-nick-translated 498 bp *AccI*/*SmaI* fragment of the protein coding region. The same transcripts were identified with a 953 bp *Hin*cII fragment of the *ypt2* 3′-untranslated region. Length markers were *Hae*III digest ϕx174 DNA and *S.cerevisiae* ribosomal RNAs.

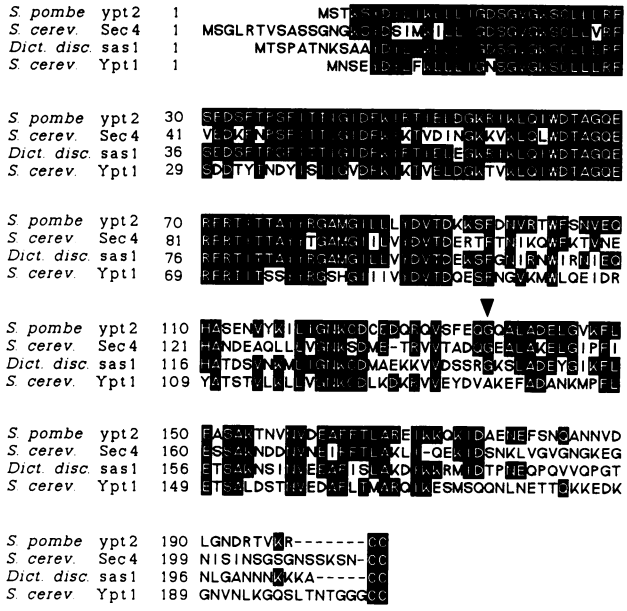

Fig. 3. Comparison of *S.pombe* ypt2 protein sequence with sequences of other ypt proteins from different species: *S.cerevisiae* Sec4p (Salminen and Novick, 1987); *Dictyostelium discoideum* sas1p (Saxe and Kimmel, 1988); *S.cerevisiae* Ypt1p (Gallwitz *et al.*, 1983). Residues identical in the ypt2 protein with those in the other proteins are displayed on dark background. An arrow points to the glycine residue which, when substituted with aspartic acid in the *S.cerevisiae* Sec4 protein, leads to a temperature-sensitive phenotype (mutation *sec4-8*). Note the conservation of serine in position 18 (with respect to *S.pombe* ypt2 protein) and of two cysteine residues at the carboxyl termini, regarded to be typical for ypt proteins (Gallwitz *et al.*, 1989; Haubruck *et al.*, 1989).

sporulation, tetrads analysed from two desired transformants gave rise to not more than two viable spores, all of which were *ura4*$^-$ (Table I). This shows that the *ypt2* gene product fulfils an essential function.

S.pombe ypt2 is the functional homologue of S.cerevisiae SEC4

An amino acid sequence comparison of *S.pombe* ypt2 with different ras-related GTP-binding proteins shows that the extent of identities is highest with the *Dictyostelium* sas1 and the *S.cerevisiae* Sec4 protein (Figure 3). Particularly noteworthy is the identical sequence of the three proteins

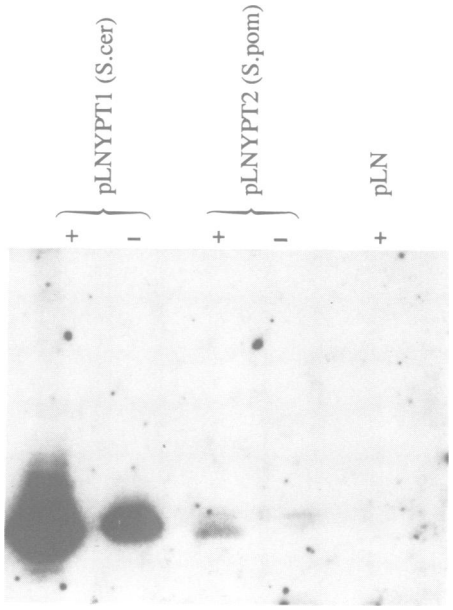

Fig. 4. GTP-blot analysis to show GTP binding of *S.pombe* ypt2 protein. *E.coli* cells transformed with either expression vector pLN or with the same vector carrying the protein-coding region of *S.cerevisiae YPT1* (pLNYPT1) or *S.pombe ypt2* (pLNYPT2) were uninduced (−) or induced with IPTG (+). Proteins of whole-cell extracts in SDS-containing buffer were separated by SDS−PAGE, transferred to a nitrocellulose filter and treated with [α-^{32}P]GTP. According to protein staining, comparable amounts of foreign proteins were present in extracts from pLNYPT1- and pLNYPT2-transformed cells analysed on the gel. Note the slightly faster electrophoretic mobility and the significantly lower capacity for GTP binding of *S.pombe* ypt2p compared to *S.cerevisiae* Ypt1p.

within a region corresponding to amino acids 32−40 in mammalian ras proteins, the so-called 'effector region' (Sigal *et al.*, 1986). This region is believed to interact wth GAP, a GTPase-activating protein (Trahey *et al.*, 1988; Vogel *et al.*, 1988). The sequence comparison is presented in Figure 7.

Because of the highly similar primary structures of the *S.pombe* ypt2 and the *S.cerevisiae* Sec4 protein, the latter of which is involved in a late step of protein secretion—the fusion of small vesicles with the plasma membrane (Goud *et al.*, 1988)—we sought to examine whether the ypt2 protein could rescue a conditional-lethal *sec4* mutant at the non-permissive temperature. For this purpose, a centromere-containing vector, YCp50-GPY2 (Figure 6), was constructed carrying the *S.pombe ypt2* protein-coding region under transcriptional control of the inducible *GAL10* promoter. A temperature-sensitive *S.cerevisiae sec4*$^-$ strain, NY405, unable to grow at temperatures higher than 33°C, was transformed with either the vector YCp50 or the recombinant plasmid YCp50-GPY2. As can be seen in Figure 6, on glucose-containing media untransformed *sec4* cells as well as *sec4* cells harbouring YCp50 or YCp50-GPY2 grew at 25°C but not at 37°C. However, when galactose was used as the sole carbon source to induce the *GAL10* promoter, only the cells expressing the *S.pombe* ypt2 protein grew well at 37°C, the non-permissive temperature. This indicates that the *S.pombe* ypt2 protein is the functional homologue of the *S.cerevisiae* Sec4 protein.

In a separate experiment, the *S.pombe ypt2* gene under transcriptional control of the *S.cerevisiae GAL10* promoter

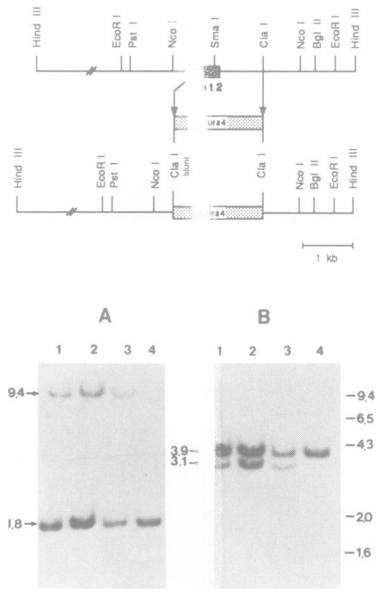

Fig. 5. Disruption of the *S.pombe* y gene. The chromosomal *Hin*dIII fragment of ~9.2 kb with r evant restriction sites and the location of the *ypt2* coding region is hown in the upper part of the figure. Schematically shown is the r lacement of the *ypt2* coding segment with a 1.8 kb *Cla*I fragmer harbouring the *S.pombe ura4* gene. A 4.75 kb *Pst*I/*Eco*RI fragme containing the *ura4* gene was used to delete one *ypt2* allele in dipl ds. Correct integration, analysed by Southern hybridization with *Hind* f-digested (**A**) and *Eco*RI/*Cla*I-digested DNA (**B**) of the three *ura4* transformants (**lanes 1–3**) and non-transformed wild-type cells (**lan 4**), is shown in the lower part of the figure. The 9.4 kb *Hin*dIII fr ment (A) and the 3.1 kb *Eco*RI/*Cla*I fragment (**B**) hybridizin o an *ura4*-specific probe have the expected length for correct integ tion. The hybridizing 1.8 kb *Hin*dIII fragment (**A**) and the 3.9 kb coRI/*Cla*I fragment (**B**) are derived from the chromosomal *ura4* ocus. Numbers to the right indicate the lengths in kb of DNA n rker fragments.

Table I. Tetrad analysis of two dipl *S.pombe* transformants after *ypt2* gene disruption

Transformant[a]	Viable:non-viable s per tetrad			pres		Ratio of *ura4*$^+$/*ura4*$^-$ spores
	4:0	3:1	2:2	:3	0:4	
UL 148-1	0	0	13		0	0/30
UL 148-2	0	0	13		0	0/28

[a]Transformants contain the *ura4* ma r gene instead of the protein-coding region of one *ypt2* al e.

was used to replace the essentia *YPT1* gene in budding yeast. High expression of the *ypt2* ne in galactose-containing medium could not rescue the *cerevisiae ypt1* null mutant (data not shown), suggesting at complementation of the *sec4* mutant by the *ypt2* gene as not simply the result of overproducing another GTP-l ding protein.

Discussion

Small GTP-binding proteins co stituting the ras superfamily are widely distributed in euka otic organisms. A primary structure comparison of dif rent members of the ras superfamily within one or b ween different eukaryotic species allows the distinction o several subfamilies, ras and ypt (rab) proteins being two them.

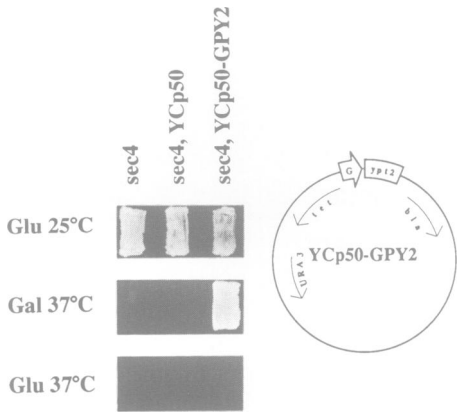

Fig. 6. Complementation by the *S.pombe ypt2* gene of a conditional-lethal *S.cerevisiae sec4* mutant. The *sec4* mutant strain GUS-1D (*sec4 ura3*), unable to grow at temperatures higher than 33°C, was transformed with either the centromere-containing plasmid YCp50 or the same vector harbouring the *S.pombe ypt2* gene under transcriptional control of the *S.cerevisiae GAL10* promoter (YCp50-GPY2). Note that at 37°C only the transformant expressing *S.pombe ypt2* gene on galactose-containing medium was able to grow.

Human	H-ras	30	D E Y D P T I E D S Y R
S.cerev.	Ras1	37	D G Y D P T I E D S Y R
S.cerev.	Ras2	37	D E Y D P T I E D S Y R
S.pombe	ras1	35	D E Y D P T I E D S Y R
Dict.disc.	ras1	30	D E Y D P T I E D S Y R
Mouse	ypt1	38	E S Y I S T I G V D F K
S.cerev.	Ypt1	35	N D Y I S T I G V D F K
S.pombe	ypt1	38	E S Y I S T I G V D F K
S.pombe	ypt2	36	P S F I T T I G I D F K
S.cerev.	Sec4	47	P S F I T T I G I D F K
Dict.disc.	sas1	42	P S F I T T I G I D F K
Dict.disc.	sas2	42	P S F I A T I G I D F K
S.pombe	ryh1	38	N T Y Q A T I G I D F L
Human	rab6	40	N T Y Q A T I G I D F L
Human	rab3A	49	P A F V S T V G I D F K
Human	rab2	33	P V H D L T I G V E F G
Human	rab4	35	D D S N H T I G V E F G
Human	rhoA	32	E V Y V P T V F E N Y V
Human	rhoB	32	E V Y V P T V F E N Y V
Human	rhoC	32	E V Y V P T V F E N Y I
Aplysia	rho	32	E V Y V P T V F E N Y V
S.cerev.	Rho1	37	E V Y V P T V F E N Y V
S.cerev.	Rho2	34	E Q Y H P T V F E N Y V

Fig. 7. Comparison of ras and ras-related proteins from different species with respect to sequence segments corresponding to the ras 'effector region' (residues 32–40 in mammalian H-ras proteins). Completely identical sequences are boxed and define highly conserved members of the ras protein superfamily. Source of protein sequence data: ras (Barbacid, 1987); mouse ypt1 (Haubruck *et al.*, 1987); *S.cerevisiae* Ypt1 (Gallwitz *et al.*, 1983); *S.pombe* ypt1 (Miyake and Yamamoto, 1990); *S.pombe* ypt2 (this paper); *S.cerevisiae* Sec4 (Salminen and Novick, 1987); *Dictyostelium discoideum* sas1 and sas2 (Saxe and Kimmel, 1988); *S.pombe* ryh1 (Hengst *et al.*, 1990); human rab (Zahraoui *et al.*, 1989); human rho (Chardin *et al.*, 1988; Yeramian *et al.*, 1987); *Aplysia* rho and *S.cerevisiae* Rho (Madaule *et al.*, 1987).

Despite the great effort in several laboratories, the cellular function of the different mammalian ras proteins has not yet been elucidated. In contrast, especially through the help of genetic analyses, the major role of the *RAS1* and *RAS2* gene products as regulators of adenylyl cyclase activity in the budding yeast *S.cerevisiae* was disclosed soon after their discovery (DeFeo-Jones *et al.*, 1983; Powers *et al.*, 1984; Toda *et al.*, 1985). Although mammalian H-ras protein

expressed in *S.cerevisiae* suppresses lethality caused by the simultaneous disruption of *RAS1* and *RAS2* genes (DeFeo-Jones *et al.*, 1985; Kataoka *et al.*, 1985), ras proteins in mammalian cells do not regulate adenylyl cyclase (Beckner *et al.*, 1985; Birchmeier *et al.*, 1985). Similarly, the protein product of the single *ras1* gene of the fission yeast *S.pombe* is not involved in cAMP metabolism (Fukui *et al.*, 1986). This seemingly complicated picture led us to initiate a study of the structure and function of ypt proteins in *S.pombe* with the final goal to investigate the generality of the model stemming from work with the very distantly related yeast *S. cerevisiae* that members of this protein family are regulators of vesicular transport processes (Bourne, 1988; Goud *et al.*, 1988; Schmitt *et al.*, 1988; Segev *et al.*, 1988).

With regard to highly conserved sequence segments typical for either ras, rho, ypt or other GTP-binding proteins (Gallwitz *et al.*, 1989), three members of the family of ypt proteins have now been identified using different approaches for their isolation. The *ypt1* and *ypt3* genes (Miyake and Yamamoto, 1990) as well as the *ypt2* gene described in this report encode different and essential functions.

As the ypt2 protein contains all the sequence motifs known to be required for guanine-nucleotide binding and GTPase activity, it was not surprising to observe GTP binding of the bacterially produced protein on GTP blots. The seemingly lower apparent capacity for GTP binding of the ypt2 protein compared to that of the *S.cerevisiae* Ypt1 protein might not be very informative as, for instance, different efficiencies of protein renaturation on the filter could cause such an effect. To this end we have not attempted to measure the nucleotide association and dissociation rate constants of the purified protein.

One of the remarkable features of the ypt2 primary structure is the region of amino acid residues 38–46 which corresponds to amino acids 32–40 in the mammalian H-ras protein, known as the 'effector region' (Sigal *et al.*, 1986) with which a GTPase-activating protein (GAP) interacts (Adari *et al.*, 1988; Calés *et al.*, 1988). This region of the *S.pombe* ypt2 protein is not only different from that of the other known GTP-binding proteins in *S.pombe*, like ypt1 and ypt3 (Miyake and Yamamoto, 1990), ras1 (Fukui and Kaziro, 1985) or ryh1 (Hengst *et al.*, 1990), but it also differs from that of the multiple ras and ras-related proteins isolated from other eukaryotes (see Figure 7). It is, however, identical to the 'effector region' of the *S.cerevisiae* Sec4 and the *Dictyostelium* sas1 protein (Salminen and Novick, 1987; Saxe and Kimmel, 1988). It was therefore of interest to find that the *S.pombe* ypt2 protein expressed in a conditional-lethal *S.cerevisiae sec4* mutant suppresses the temperature-sensitive phenotype. Together with the finding of functional interchangeability of ypt1 proteins from mouse, *S.pombe* and *S.cerevisiae* (Haubruck *et al.*, 1989; Miyake and Yamamoto, 1990) on the one hand, mammalian H-ras and *S.cerevisiae* Ras1, Ras2 (DeFeo-Jones *et al.*, 1985; Kataoka *et al.*, 1985) as well as human rab6 and *S.pombe* ryh1 (Hengst *et al.*, 1990) on the other, each group of proteins sharing identical 'effector loops' (Figure 7), this indicates that the 'effector region' is an important determinant of the functional specificity of small GTP-binding proteins. In addition, the strict evolutionary conservation of this sequence motif within groups of members of the superfamily of ras proteins also points to the conservation of proteins interacting with different GTP-binding proteins. Evidence for this assumption is the recent iscovery in *S.cerevisiae* of a gene, *IRA1*, whose prote ı product shares some homology with mammalian GAI and is integrated in the pathway regulated by Ras protei ı (Tanaka *et al.*, 1989). Interestingly, mammalian GAP is ble to complement *ira1*$^-$ yeast mutants, suggesting commo functional properties of these two proteins (Ballester *et a* ., 1989).

Our observation that the *.pombe* ypt2 protein complements a *S.cerevisiae sec4* ı utant indicates, but does not prove, that the *S.pombe* prot in acts as a regulator in intracellular protein transport. Th : existence in the fission yeast of a Sec4p homologue is of special interest, since no such protein has so far been fou ıd within the more than 20 mammalian ras-related protei ı s. As a perspective, the comparative studies on the functi ɔning of different GTP-binding proteins in distantly related eukaryotes amenable also to genetic analysis might prove to be extremely rewarding.

Materials and methods

Cloning and sequence analysis of the ypt2 gene

Total DNA from *S.pombe* strain 972 h^{-s}, digested with the restriction endonucleases *Bam*HI, *Eco*RI or *Hin*dIII, was separated in a 1% agarose gel and transferred to nitrocellulose. Hybridization was performed under moderately stringent conditions (6 × SSC, 60°C) with a ^{32}P-nick-translated 528 bp *Eco*RI–*Hin*cII fragment of the coding region of the cloned *YPT1* gene of *S.cerevisiae* (Gallwitz *et al.*, 1983).

DNA fragments of the *Hin*dIII digest in the region of the gel giving a hybridization signal (~9 kb) were eluted from a preparative agarose gel and cloned into pUC8 (Vieira and Messing, 1982). Recombinant plasmids were transformed into *E.coli* and colony screening was performed using hybridization fragment and conditions as described above.

The 9.2 kb *Hin*dIII fragment of the recombinant plasmid pPYPT1 was shown to contain a 2.65 kb cross-hybridizing *Nco*I fragment, which was subcloned into pUC8 (pPYPT11). Starting from restriction sites located in the polylinker region and in the cloned DNA fragment, the *ypt2* gene was sequenced by the method of Maxam and Gilbert (1980).

Expression of the ypt2 protein in E.coli

For the bacterial expression of ypt2 protein in *E.coli*, *Nde*I and *Bss*HII restriction sites were introduced at the 5′ and 3′ ends respectively of the *ypt2* coding region. After generating blunt ends, 2.65 kb *Nco*I fragment was cloned into the *Hin*cII site of vector pT7T3 (Pharmacia). The *Nde*I site was introduced by oligonucleotide-directed mutagenesis according to Nakamaye and Eckstein(1986) with the oligonucleotide 5′-GGATTT-TGTAGACATATGGGAGTGTTGC-3′. The *Bss*HII site was likewise generated by the oligonucleotide 5′-CAAGATAATAATAGCGCGCCT-AACAACAC-3′ . The coding region of the *ypt2* gene as a 612 bp *Nde*I–*Bss*HII fragment was then used to replace the coding region of the *S.cerevisiae YPT1* gene (a 626 bp *Nde*I/*Bss*HII fragment) in the vector pLNYPT1. Expression of ypt2 protein in *E.coli* and nucleotide-binding analysis were done as described previously (Wagner *et al.*, 1987).

Disruption of the ypt2 gene

The 1.6 kb *Acc*I–*Cla*I fragment containing the *ypt2* gene in plasmid pPYPT1 was replaced in several steps by a 1.8 kb *Hin*dIII fragment of pSUC1-DI harbouring the *S.pombe ura4* marker gene.

For *ypt2* gene disruption, *S.pombe* strain UL130 (*h*$^+$/*h*$^-$ *ade6-M210/ade6-M216 ura4-294/ura4-294*) was transformed by the lithium acetate method (Ito *et al.*, 1983) with a 4.75 kb *Pst*I/*Hin*dIII fragment of the *ypt2* locus carrying the *ura4* gene instead of the *ypt2* coding region. Correct integration in *ura4*$^+$ transformants (strains UL148: *h*$^+$/*h*$^-$ *ade6-M210/ade6-M216 ura4-294/ura4-294 ypt2*$^+$/*ypt2::ura4*$^+$) was verified by Southern analysis with either *Hin*dIII or *Eco*RI/*Cla*I-digested DNA.

Complementation of S.cerevisiae sec4

To complement the temperature-sensitive phenotype of *sec4* strain NY405 (Salminen and Novick, 1987), a centromere plasmid was constructed that contained the *ypt2* gene under the control of the *GAL10* promoter. From the recombinant plasmid pEV-Galmypt1 (Haubruck *et al.*, 1989), a 580 bp *Bgl*II/*Nde*I fragment containing the *Gal10* promoter sequence and a 448 bp

*Bss*HII/*Xho*I fragment with 188 bp of 3′-untranslated region of the *S. cerevisiae YPT1* gene were ligated together with the 612 bp *Nde*I/*Bss*HII fragment of the *ypt2* coding region (see above) and cloned into the *Hin*dIII site of YCp50 (Rose *et al.*, 1987) to yield plasmid YCp50-GPY2. As the *S.cerevisiae* strain NY405 (*MATa sec4-8 ura3-52*) exhibited poor growth in galactose-containing media, it was crossed with AG430-9D *MATα leu2 his3*) to create the diploid strain GUS. Sporulation and tetrad analysis yielded one spore GUS-1D (*MATα sec4-8, ura3-52*), which grew well on galactose as the sole carbon source. Strain GUS-1D was transformed with either plasmid YCp50 or YCp50-GPY2. Six out of eight tested transformants carrying YCp50-GPY2 were able to grow on galactose- but not on glucose-containing media at 37°C, whereas cells transformed with YCp50 were unable to grow at 37°C regardless of the carbon source.

Other methods

Preparation of DNA and RNA, Northern and Southern blots were as described previously (Langford and Gallwitz, 1983; Langford *et al.*, 1984). All media used and general genetic manipulations of *S.pombe* have been described elsewhere (Gutz *et al.*, 1974). For the isolation of diploid strains, the procedures of Flores da Cunha (1970) were used. Growth of *S.cerevisiae* cells, strain construction, sporulation of diploids, tetrad analysis and scoring of genetic markers were performed by standard methods (Sherman *et al.*, 1986).

Acknowledgements

We are indebted to Herbert Gutz (Braunschweig) and Peter Novick (New Haven) for providing some of the yeast strains used in this study. We thank Masayuki Yamamoto (Tokyo) for helpful discussions and for exchanging sequence information before publication. We also thank Hans-Peter Geithe for synthesizing oligonucleotides, Heike Behr for competent technical assistance and Ingrid Balshüsemann for invaluable secretarial help. Part of this work was supported by grants to D.G. of the Bundesministerium für Forschung und Technologie (BMFT) and the Fonds der Chemischen Industrie.

References

Adari,H., Lowy,D.R., Willumsen,B.M., Der,C.J. and McCormick,F. (1988) *Science,* **240**, 518–521.

Baker,D., Wuestehube,L., Scheckman,R., Botstein,D. and Segev,N. (1990) *Proc. Natl. Acad. Sci. USA,* **87**, 355–359.

Ballester,R., Michaeli,T., Ferguson,K., Xu,H.-P., McCormick,F. and Wigler,M. (1989) *Cell,* **59**, 681–686.

Barbacid,M. (1987) *Annu. Rev. Biochem.*, **56**, 779–827.

Beckner,S.K., Hattori,S. and Shih,T.Y. (1985) *Nature,* **317**, 71–72.

Birchmeier,C., Broek,D. and Wigler,M. (1985) *Cell,* **43**, 615–621.

Bourne,H.R. (1988) *Cell,* **53**, 669–671.

Calés,C., Hancock,J.H., Marshall,C,J. and Hall,A. (1988) *Nature,* **332**, 548–551.

Chardin,P. (1988) *Biochimie,* **70**, 865–868.

Chardin,P., Madaule,P. and Tavitian,A. (1988) *Nucleic Acids Res.*, **16**, 2717.

DeFeo-Jones,D., Scolnick,E.M., Koller,R. and Dhar,R. (1983) *Nature,* **306**, 707–709.

DeFeo-Jones,D., Tatchell,K., Robinson,L.C., Sigal,I.S., Vass,W.C., Lowy,D.R. and Scolnick,E.M. (1985) *Science,* **228**, 179–184.

Dever,T.E., Glynias,M.J. and Merrick,W.C. (1987) *Proc. Natl. Acad. Sci. USA,* **84**, 1814–1818.

De Vos,A.M., Tong,L., Milburn,M.V., Matias,P.M., Jangarik,J., Noguchi,S., Nishimura,S., Miura,K., Ohtsuka,E. and Kim,S.-H. (1988) *Science,* **239**, 888–893.

Fawell,E., Hook,S. and Armstrong,J. (1989) *Nucleic Acids Res.*, **17**, 4373.

Flores da Cunha,M. (1970) *Genet. Res.*, **16**, 127–144.

Fukui,Y. and Kaziro,Y. (1985) *EMBO J.*, **4**, 687–691.

Fukui,Y., Kozasa,T., Kaziro,Y., Takeda,T. and Yamamoto,M. (1986) *Cell,* **44**, 329–336.

Gallwitz,D., Donath,C. and Sander,C. (1983) *Nature,* **306**, 704–707.

Gallwitz,D., Haubruck,H., Molenaar,C., Prange,R., Puzicha,M., Schmitt,H.D., Vorgias,C. and Wagner,P. (1989) In Bosch,L., Kraal,B. and Parmeggiania,A. (eds), *The Guanine-nucleotide Binding Proteins.* Plenum Press, New York, NATO ASI Series A: Life Sciences, Vol. 165, pp. 257–264.

Goud,B., Salminen,A., Walworth,N.C. and Novick,P.J. (1988) *Cell,* **53**, 753–768.

Gutz,H., Heslot,H., Leupold,U. and Loprieno,N. (1974) In King,R.D. (ed.), *Handbook of Genetics.* Plenum Press, New York, Vol. 1, pp. 395–446.

Haubruck,H., Disela,C., Wagner,P. and Gallwitz,D. (1987) *EMBO J.*, **6**, 4049–4053.

Haubruck,H., Prange,R., Vorgias,C. and Gallwitz,D. (1989) *EMBO J.*, **8**, 1427–1432.

Hengst,L., Lehmeier,T. and Gallwitz,D. (1990) *EMBO J.*, **9**, 1949–1955.

Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) *J. Bacteriol.*, **153**, 163–168.

Kataoka,T., Powers,S., Cameron,S., Fasano,O., Goldfarb,M., Broach,J. and Wigler,M. (1985) *Cell,* **40**, 19–26.

Langford,C.J. and Gallwitz,D. (1983) *Cell,* **33**, 519–527.

Langford,C.J., Klinz,F.-J., Donath,C. and Gallwitz,D. (1984) *Cell,* **36**, 645–653.

Madaule,P., Axel,R. and Myers,A.M. (1987) *Proc. Natl. Acad. Sci. USA,* **84**, 779–783.

Maxam,A. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–560.

Miyake,S. and Yamamoto,M. (1990) *EMBO J.*, **9**, 000–000.

Molenaar,C.M.T., Prange,R. and Gallwitz,D. (1988) *EMBO J.*, **7**, 971–976.

Nakamaye,K.L. and Eckstein,F. (1986) *Nucleic Acids Res.*, **14**, 9679–9698.

Pai,E.F., Kabsch,W., Krengel,U., Holmes,K.C., John,J. and Wittinghofer,A. (1989) *Nature,* **341**, 209–214.

Powers,S., Kataoka,T., Fasano,O., Goldfarb,M., Strathern,J., Broach,J. and Wigler,M. (1984) *Cell,* **36**, 607–612.

Rose,M.D., Novick,P., Thomas,J.H., Botstein,D. and Fink,G.R. (1987) *Gene,* **60**, 237–243.

Russel,P. and Nurse,P. (1986) *Cell,* **45**, 781–782.

Salminen,A. and Novick,P.J. (1987) *Cell,* **49**, 527–538.

Saxe,S.A. and Kimmel,A.R. (1988) *Dev. Genet.*, **9**, 259–265.

Schmitt,H.D., Wagner,P., Pfaff,E. and Gallwitz,D. (1986) *Cell,* **47**, 401–412.

Schmitt,H.D., Puzicha,M. and Gallwitz,D. (1988) *Cell,* **53**, 635–647.

Segev,N., Mulholland,J. and Botstein,D. (1988) *Cell,* **52**, 915–924.

Sherman,F., Fink,G.R. and Hicks,J.B. (1986) *Laboratory Course Manual for Methods in Yeast Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sigal,I.S., Gibbs,J.B., D'Alonzo,J.S., Temeles,G.L., Wolanski,B.S., Socher,S.H. and Scolnick,E.M. (1986) *Proc. Natl. Acad. Sci. USA,* **83**, 952–956.

Tanaka,K., Matsumoto,K. and Toh,E.-A. (1989) *Mol. Cell. Biol.*, **9**, 757–768.

Toda,T., Uno,I., Ishikawa,T., Powers,S., Kataoka,T., Broek,D., Cameron,S., Broach,J., Matsumoto,K. and Wigler,M. (1985) *Cell,* **40**, 27–36.

Touchot,N., Chardin,P. and Tavitian,A. (1987) *Proc. Natl. Acad. Sci. USA,* **84**, 8210–8214.

Trahey,M., Wong,G., Halenbeck,R., Rubinfeld,B., Martin,G., Ladner,M., Long,C.M., Crosier,W.J., Watt,K., Koths,K. and McCormick,F. (1988) *Science,* **242**, 1697–1700.

Vieira,J. and Messing,J. (1982) *Gene,* **19**, 259–268.

Vogel,U.S., Dixon,R.A.F., Schaber,M.D., Diehl,R.E., Marshall,M.S., Scolnick,E.M., Sigal,I.S. and Gibbs,J.B. (1988) *Nature,* **335**, 90–93.

Wagner,P., Molenaar,C.M.T., Rauh,A.J.G., Bröckel,R., Schmitt,H.D. and Gallwitz,D. (1987) *EMBO J.*, **6**, 2373–2379.

Walworth,N.C., Goud,B., Kabcenell,A.K. and Novick,P.J. (1989) *EMBO J.*, **8**, 1685–1693.

Yeramian,P., Chardin,P., Madaule,P. and Tavitian,A. (1987) *Nucleic Acids Res.*, **15**, 1869.

Zahraoui,A., Touchot,N., Chardin,P. and Tavitian,A. (1989) *J. Biol. Chem.*, **264**, 12394–12401.

Received on January 17, 1990; revised on February 26, 1990