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The *ryh1* gene in the fission yeast *Schizosaccharomyces pombe* encoding a GTP-binding protein related to ras, rho and ypt: structure, expression and identification of its human homologue

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A gene, *ryh1*, of the fission yeast *Schizosaccharomyces pombe* encoding a GTP-binding protein of 201 amino acids and belonging to the ras superfamily was isolated using the protein-coding region of the cloned *Saccharomyces cerevisiae YPT1* gene as hybridization probe. The *ryh1* gene is interrupted by three introns. *ryh1* null mutants are viable but unable to grow at temperatures >35.5°C. Invertase of *ryh1*$^-$ cells is properly secreted but has a faster electrophoretic mobility compared to that of wild-type cells. The temperature-sensitive phenotype of *ryh1* null mutants is complemented by the human *rab6* cDNA expressed either under transcriptional control of the *S.pombe adh* or the SV40 early promoter.

Key words: GTP-binding proteins/H-rab6 homologue/ras superfamily/*ryh1* gene/*Schizosaccharomyces pombe*

Introduction

From the intense studies of 'classical' G-proteins, which as heterotrimers are mediators of signal transduction in eukaryotes between membrane-bound receptors and different effector proteins (for review see Gilman, 1987), it is a widely held assumption that GTP/GDP-binding, regulatory proteins serve to amplify ligand-induced signals of various kinds. Although this might also be true for the monomeric ras proteins (for review see Barbacid, 1987), and it is almost certainly correct for the *RAS* gene products in yeast (for review see Wigler *et al.*, 1988), a different view has been taken regarding the functioning of various ras-related proteins. In this hypothesis, which is mainly based on functional studies of ras-like proteins in the yeast *Saccharomyces cerevisiae*, GTP-binding proteins, in analogy to the well-understood role of elongation factors in protein synthesis, serve to regulate unidirectional transport processes (for review see Bourne, 1988). Through the advance of DNA cloning and sequencing, an unexpectedly large number of structurally related and evolutionary highly conserved, small GTP-binding proteins has been discovered within the past few years. They can be viewed as members of the ras superfamily of proteins and further classified as ras, rho, ypt (rab) and arf proteins (for review see Botstein *et al.*, 1988; Chardin *et al.*, 1989; Gallwitz *et al.*, 1989).

Two members of the ypt protein family in the budding yeast *S.cerevisiae*, the Ypt1 protein, Ypt1p (Gallwitz *et al.*, 1983), and the Sec4 protein, Sec4p (Salminen and Novick, 1987), seem to play a role in the directed transport of vesicles between different compartments of the secretory pathway (Goud *et al.*, 1988; Schmitt *et al.*, 1988; Segev *et al.*, 1988; Walworth *et al.*, 1989; Baker *et al.*, 1990). These studies were aided by the easy accessibility of these unicellular organisms to classical and molecular genetic methods, such as gene disruption and replacement and the creation and analysis of conditional-lethal mutants.

Although the high conservation of ras and ras-related proteins in yeast and mammals suggests similar functions of these proteins in different eukaryotes, the pathways regulated by ras proteins in yeasts and mammals are not identical (Beckner *et al.*, 1985; Birchmeier *et al.*, 1985; Fukui *et al.*, 1986). This situation led us and others to a search for ras-like proteins and their function in the fission yeast *Schizosaccharomyces pombe*, a genetically tractable unicellular organism that, in many respects (e.g. cell cycle events, chromosome and gene structure, morphology of cellular structures), resembles higher eukaryotes more than *S.cerevisiae*. In the accompanying papers of Miyake and Yamamoto (1990) and Haubruck *et al.* (1990), three members of the ypt gene family, *ypt1*, *ypt2* and *ypt3*, are described whose protein products serve different cellular functions and are able to replace the essential *S.cerevisiae* Ypt1p (*S.pombe* ypt1p) or Sec4p (*S.pombe* ypt2p). Here we report the isolation of yet another gene encoding a ras-related protein that we have designated ryh1. Although there seems to exist only one *ryh* gene in *S.pombe*, the disruption of *ryh1* is not lethal but leads to a temperature-sensitive (ts) phenotype. By complementation of the ts phenotype we were able to identify the human rab6 protein (Zahraoui *et al.*, 1989) as the likely functional homologue of ryh1p.

Results

Cloning and sequence of ryh1

The strategy for cloning the gene was identical to that employed to isolate the *ypt2* gene. As described in the accompanying paper (Haubruck *et al.*, 1990), *Hin*dIII-digested *S.pombe* DNA, when probed with a *S.cerevisiae YPT1* gene fragment, revealed three major hybridizing fragments of ~9, 3 and 0.5 kb. From a preparative agarose gel, DNA fragments between 2 and 4 kb were isolated, inserted into *Hin*dIII-cut pUC8 and cloned in *Escherichia coli*. The recombinant plasmid, pPYPT-T, isolated from several positive clones, contained a 3.1 kb *Hin*dIII fragment which hybridized back to the *YPT1*-specific probe. After mapping the cloned DNA fragment with different restriction endonucleases, the sequence of ~1700 bp was determined by the method of Maxam and Gilbert.

Taking advantage of the known conserved sequence motifs of GTP-binding proteins to search for a reading frame, a gene encoding a protein of 201 amino acids, interrupted by three short introns, was detected (Figure 1). The introns could be identified by their characteristic

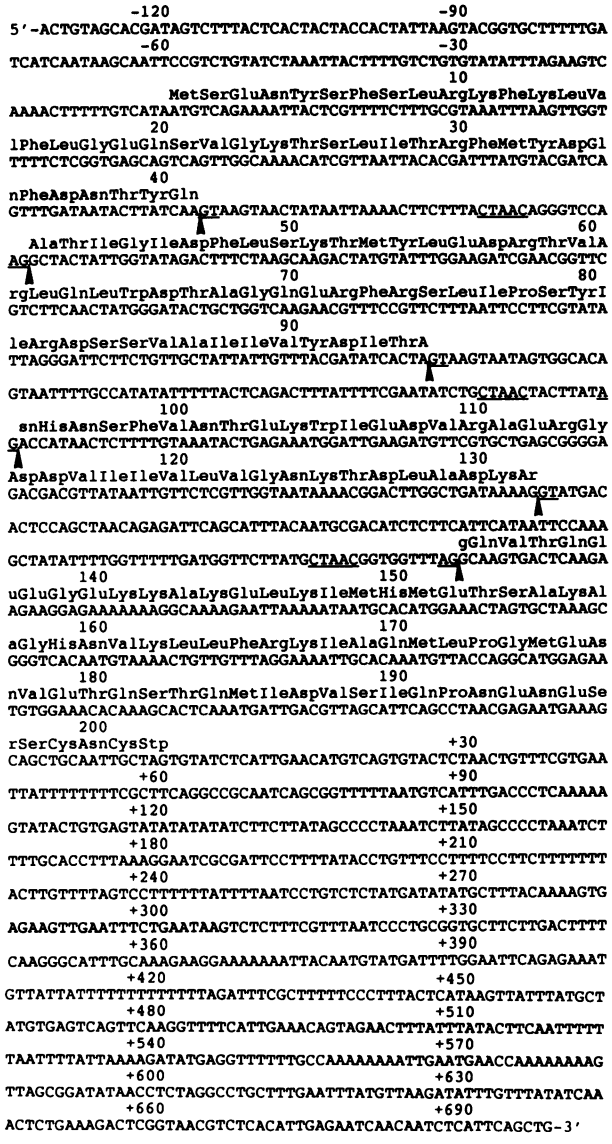

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the *S.pombe ryh1* gene. Intron−exon junctions are indicated by arrows. Conserved intron sequences are underlined. Restriction enzyme recognition sites used for deleting part of the gene and the insertion of the *ura4* gene in the gene disruption experiments are located at codons 92/93 (*Eco*RV) and at base pairs +173 to 178 (*Dra*I) and +183 to 188 (*Nru*I). The *S.pombe ryh1* gene has been given the accession number X52475 in the EMBL Data Library.

sequences at the 5′ splice site, 5′-GTAXG-3′, the 3′ splice site, 5′-$^{T}/_{A}$AG-3′, and the branch-site, 5′-CTPuAPy-3′ (Mertins and Gallwitz, 1987; Gallwitz *et al.*, 1988). The predicted reading frame was verified by sequence determination of a complete cDNA. This was obtained by PCR amplification from a *S.pombe* cDNA library with the help of two primers complementary to sequences flanking the protein-coding part of the *ryh1* gene. The nucleotide sequence of the protein-coding region of the cloned gene was completely identical with that of the cDNA.

The ryh1 gene encodes a GTP-binding protein

As deduced from the DNA sequence, the *ryh1* gene has the coding capacity for a protein of 201 amino acid residues and a molecular mass of 23 110 daltons. The four regions required for nucleotide binding, characteristic in sequence

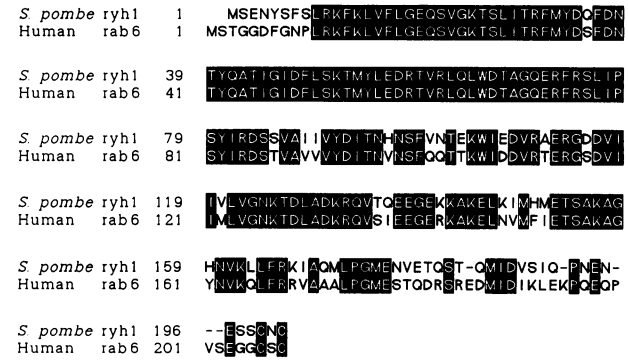

Fig. 2. Comparison of primary structures of the *S.pombe* ryh1 and the human rab6 protein. Identical amino acids are on dark background. The H-rab6 sequence is from Zahraoui *et al.* (1989).

and spacing in all ras and ras-like proteins, are found in the ryh1 protein. These sequence segments, **GEQSVGKT** (amino acids 18−25), **DTAG** (amino acids 66−69), **NKTD** (amino acids 124−127) and **ETSA** (amino acids 152−155), with bold letters indicating highly conserved amino acid residues, are indeed completely identical with those in the human rab6 protein (Zahraoui *et al.*, 1989), the member of the ras superfamily that we find to be most closely related to the *S.pombe* ryh1 protein. Allowing for only five gaps in the region of the carboxyl-terminal 23 amino acids, the two proteins are identical to a degree of 73% (Figure 2). Noteworthy also are the cysteine-coding C termini of the two proteins, -Cys-Asn-Cys (ryh1) and -Cys-Ser-Cys (H-rab6).

The capacity of the ryh1 protein for GTP binding was analysed on protein blots from bacteria producing the yeast protein. For this purpose, the *ryh1* cDNA as a 618 bp *Nde*I−*Dra*I fragment under *lacZ* promoter control had been inserted into the expression vector pLN (Wagner *et al.*, 1987). As can be seen in Figure 3, *E.coli* transformed with the vector pLNryh1, on induction with IPTG produces a protein similar in size to the *S.cerevisiae* Ypt1 protein and which is also able to bind GTP.

Phenotypic alterations following ryh1 gene disruption

In a first attempt to study the physiological role of the *ryh1* gene product in yeast cells, a *ryh1* null mutant was constructed by deleting from the gene a 696 bp *Eco*RV−*Dra*I fragment containing codons 93−201, the stop codon and 175 bp of the 3′-non-translated region. The *S.pombe ura4* gene, inserted as a 1.75 kb *Hin*dIII fragment into the *Nru*I restriction site 8 bp 3′ of the deletion end point, served as a marker for successful transformation and correct integration into the genome of a diploid strain lacking both *ura4* alleles (Figure 4). Of 12 stable integrants analysed, 11 had one *ryh1* gene disrupted through homologous recombination with the 4.2 kb *Hin*dIII fragment used for transformation.

This is documented by the Southern analysis of Figure 4(B) in which *Eco*RI-digested DNA of 12 transformants (lanes 1−12) and from untransformed yeast (lane 13) was probed with the ^{32}P-labelled *Hin*dIII fragment used for transformation. In addition to a 1.6 and a 5.2 kb fragment (wild-type), 11 transformants displayed the expected hybridizing *Eco*RI fragment of ~6.2 kb (lanes 1−11). Hybridization with a *ura4*-specific probe led to significant

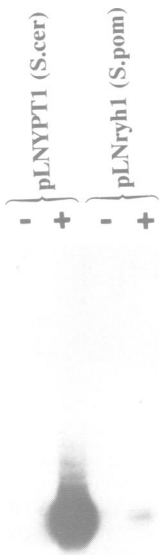

Fig. 3. GPT binding of bacterially produced ryh1 protein. *E.coli* transformed with the expression plasmid pLNYPT1 or pLNryh1 was grown in the absence (−) or presence (+) for 2 h of 1 mM IPTG to induce the synthesis of the foreign proteins. Total bacterial protein was separated by SDS−PAGE, transferred electrophoretically to a nitrocellulose filter and probed for binding of [α-^{32}P]GTP as previously described (Wagner *et al.*, 1987). Comparable amounts of *S.cerevisiae* Ypt1p and *S.pombe* ryh1p, as judged by staining with Coomassie Brilliant Blue, were produced in the two transformants. Note the significantly lower apparent GTP-binding capacity of the ryh1p compared to Ypt1p.

signals of only the 6.2 kb fragments (data not shown), since the *ura4* genes were deleted from the strain used for transformation. The additional hybridizing fragment of the transformant analysed in lane 12 (Figure 4B) could be the result of an additional, unwanted integration event.

Examination of tetrads from several strains with one *ryh1* wild-type and one null allele showed that *ryh1* is not essential for cell viability. Tetrads uniformly gave rise to two large and two small colonies (Figure 4C), the small colonies being of *ura4*$^+$ phenotype. On inoculation into liquid medium, *ryh1*$^-$ cells were characterized by a somewhat extended lag phase, but after attainment of logarithmic growth at 30°C, the generation times of wild-type and mutant cells differed only slightly. However, at temperatures >35.5°C, *ryh1*$^-$ cells were unable to grow (Figure 5). To show unequivocally that the *ryh1* gene was disrupted in the *ura4*$^+$ ts haploids, RNA was prepared from the progeny of four spores derived from a complete tetrad and subjected to Northern blot analysis. As shown in Figure 6, in *ura4*$^-$, *ryh1*$^+$ cells ('wild-type') but not in the *ura4*$^+$, *ryh1*$^-$ cells three transcripts with a length of ~1600, 1800 and 2100 nucleotides respectively, hybridized to a ^{32}P-labelled 485 bp *Eco*RI−*Nru*I fragment isolated from an incomplete cDNA and encompassing the *ryh1* protein-coding sequence from codons 102 to 201 and 185 bp of the 3′-untranslated region. As this fragment was part of the deleted sequence in the construction used for gene disruption, the result of the RNA blot analysis demonstrated that (i) the ts cells lacked a functional *ryh1* gene and (ii) all three hybridizing RNA species were transcripts of the same gene.

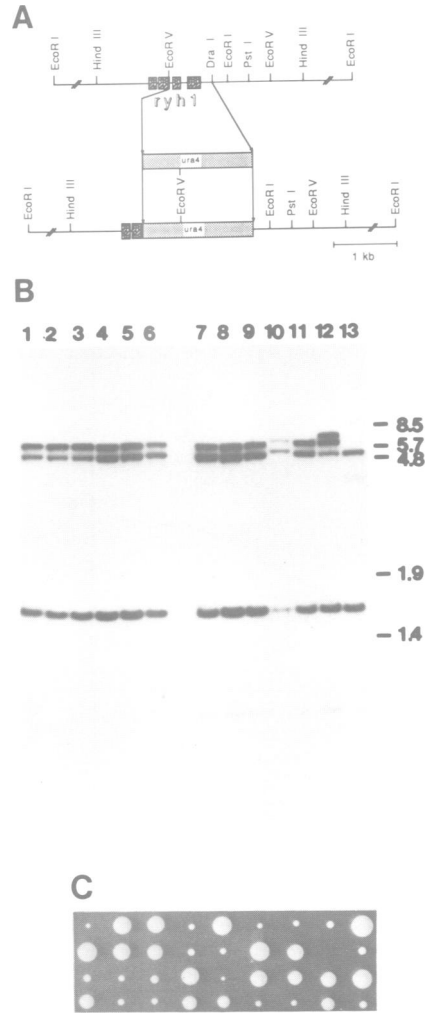

Fig. 4. Disruption of the *ryh1* gene. (**A**) Schematic representation of the *ryh1* locus with relevant restriction sites and of the deletion of an *Eco*RV—*Dra*I fragment containing part of the *ryh1*-coding region. The *S.pombe ura4* gene on a 1.75 kb *Hin*dIII fragment was inserted into the *Nru*I restriction site 8 bp 3′ of the *Dra*I site. (**B**) Southern analysis to verify correct chromosomal integration of the disrupted gene on a 4.2 kb *Hin*dIII fragment. *Eco*RI-digested DNA from 12 *ura4*$^+$ transformants (**lanes 1−12**) and from the untransformed strain (**lane 13**) was probed with the ^{32}P-labelled 4.2 kb *Hin*dIII fragment used for transformation. The appearance of one additional hybridizing 6.2 kb fragment (**lanes 1−11**) indicates correct integration and disruption of one *ryh1* allele. The positions of length marker fragments (in kb) are shown to the right. (**C**) Tetrad analysis of the *ryh1*$^-$/*ryh1*$^+$ diploid analysed in **lane 6** (B). Spores that were *ura4*$^+$ and *ryh1*$^-$ generate the small colonies.

In the budding yeast *S.cerevisiae* two proteins of the ypt branch of the ras superfamily, the Ypt1 and the Sec4 protein, have been shown to be involved in intracellular protein transport (Goud *et al.*, 1988; Schmitt *et al.*, 1988; Segev *et al.*, 1988; Walworth *et al.*, 1989; Baker *et al.*, 1990). Conditional-lethal *ypt1* mutants are defective in invertase secretion and accumulate core-glycosylated invertase intracellularly (Schmitt *et al.*, 1988; Segev *et al.*, 1988). We examined the distribution of invertase in *ryh1*$^+$ and *ryh1*$^-$ *S.pombe* strains to see whether the lack of this GTP-binding protein interfered with a process in protein transport from the endoplasmic reticulum (ER) to the plasma membrane. As previously reported (Moreno *et al.*, 1985), *S.pombe* invertase has a significantly higher apparent mol. wt than

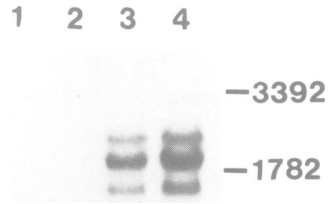

Fig. 5. Northern blot analysis with total cellular RNA from two *ryh1*$^-$ (**lanes 1** and **2**) and two *ryh1*$^+$ strains (**lanes 3** and **4**) derived from a complete tetrad shown in Figure 4(C). The position and length (in nucleotides) of rRNAs from *S.cerevisiae* separated on the same gel are shown to the right.

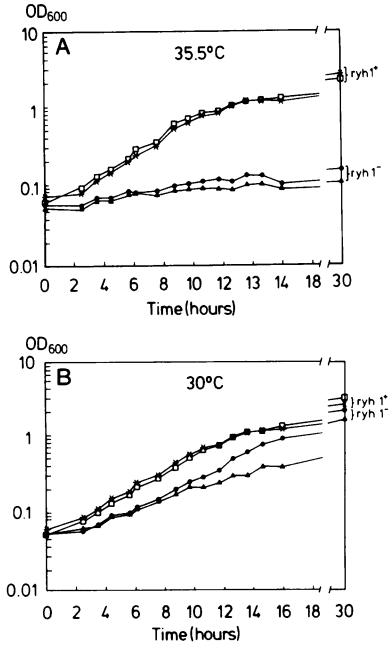

Fig. 6. Growth curves of two *ryh1*$^+$ and two *ryh1*$^-$ strains derived from a complete tetrad (Figure 4C). Cells logarithmically growing at 30°C were diluted to approximately the same optical density, and half of the cultures were further grown at either 30 or 35.5°C. At the times indicated, aliquots were taken to measure the optical density at 600 nm.

the secreted form of invertase in *S.cerevisiae*, and it is localized predominantly outside the plasma membrane. The presence of external and cytosolic invertase was examined through the generation and lysis of spheroplasts from *ryh1*$^+$ and *ryh1*$^-$ cells derived from a complete tetrad and grown at 30°C. After electrophoresis under non-denaturing conditions, active invertase, identified by staining in a 3.5% polyacrylamide gel, could be detected in the fraction of proteins that were ouside the plasma membrane but not in the cytoplasm. Although no intracellular accumulation of invertase in *ryh1*$^-$ cells was observed, the enzyme from cells lacking this GTP-binding protein exhibited a faster electrophoretic mobility (Figure 7). This might point to an underglycosylation of invertase in *ryh1*$^-$ mutants. No major difference in the localization and electrophoretic behaviour of invertase was observed with *ryh1* null mutants grown at 30 or 36°C (data not shown).

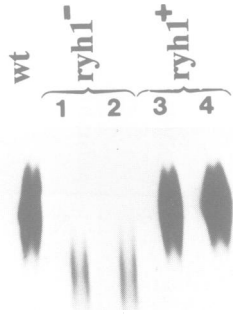

Fig. 7. Electrophoretic analysis of secreted, enzymatically active invertase. Proteins solubilized during zymolase digestion of cells that had been grown logarithmically at 30°C were separated on a 3.5% polyacrylamide gel and assayed for active enzyme as described by Moreno *et al.* (1985). A diploid strain used for *ryh1* gene disruption (wt) and haploids that were either *ryh1*$^+$ (**lanes 3** and **4**) or *ryh1*$^-$ (**lanes 1** and **2**) and derived from one tetrad (Figure 4C) were derepressed for invertase synthesis in medium containing 0.1% glucose for 3 h before analysis.

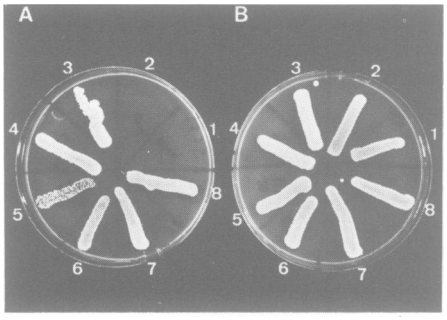

Fig. 8. Complementation of the ts phenotype of *ryh1*$^-$ cells by the human rab6 protein. *ryh1*$^-$ (**1,2**) and *ryh1*$^+$ haploids (**3,4**) derived from a tetrad of a diploid with one disrupted *ryh1* allele and two *ryh1*$^-$ transformants each, expressing the human *rab6* cDNA on a plasmid either under the control of the SV40 early promoter (**5,6**) or the *S.pombe adh* promoter (**7,8**) were spread onto agar plates and incubated at either 30°C (**B**) or 36°C (**A**).

Human rab6 complements ts ryh1$^-$ *mutants*

As pointed out above, the primary structure of the ryh1 protein is highly related to that of the human rab6 protein. Particularly noteworthy is the completely identical sequence of 26 and 49 consecutive amino acids in positions 9–36 and 38–84 (with respect to the *S.pombe* ryh1 protein, Figure 2). The second block of identical amino acids contains the 'effector loop' (amino acids 40–48 and 42–50 in the ryh1 and H-rab6 protein respectively) analogous to the GAP-binding region of ras protein (for review see McCormick, 1989).

As several distinct GTP-binding proteins from evolutionarily very distant species are functionally interchangeable, we sought to examine whether the ts phenotype resulting from *ryh1* gene disruption could be suppressed by the human rab6 protein. The *rab6* cDNA (kindly provided by A.Tavitian) was brought under transcriptional control of either the *S.pombe adh* or the SV40 early promoter, inserted into yeast/*E.coli* shuttle vectors pEVP11 or pSM2 to obtain the

recombinant plasmids pADH-rab6 and pSV40-rab6. These plasmids were used to transform a *ryh1*$^-$ mutant strain. As shown in Figure 8, *ryh1*$^-$ cells, unable to grow at 36°C, grew well at this temperature when expressing the human rab6 protein regardless of the promoter driving the human cDNA transcription. It should be noted that SV40 promoter-driven genes in *S.pombe* are only moderately overexpressed, whereas genes under *S.pombe adh* promoter control are very efficiently transcribed (Moreno *et al.*, 1990). Transformation of *ryh1*$^-$ mutant cells with the same vectors lacking the *rab6* cDNA did not suppress the ts phenotype (data not shown). This study indicates that the human rab6 protein is a functional homologue of the *S.pombe* ryh1 protein.

Discussion

We have described the isolation and structure of a gene in *S.pombe* that encodes a protein belonging to the ras superfamily. The protein contains the four conserved sequence elements $GX_4GK^S/_T$, DX2G, NKXD and EXSA (for review see Barbacid, 1987; Dever *et al.*, 1987; Gallwitz *et al.*, 1989) known to interact with the bound guanine nucleotide (De Vos *et al.*, 1988; Pai *et al.*, 1989). We have previously argued (Haubruck *et al.*, 1987; Gallwitz *et al.*, 1989) that characteristically conserved sequences adjacent to the nucleotide-binding motifs allow one to distinguish at least three families among the ras and ras-related proteins which are known as ras (for review see Barbacid, 1987), rho (Madaule *et al.*, 1987) and ypt proteins (Gallwitz *et al.*, 1983, 1989). Mammalian ypt proteins have also been designated rab (Touchot *et al.*, 1987; Zahraoui *et al.*, 1989) or smg (Matsui *et al.*, 1988). Still another family, that of the arf proteins (Botstein *et al.*, 1988; Sewell and Kahn, 1988), could be considered to belong to the ras superfamily of proteins.

As the newly discovered protein, although most closely related to ypt, does not fit all of these structural criteria, we have provisionally named it ryh1 (for **r**as/**r**ho/**y**pt **h**omologue). The remarkable evolutionary conservation of many of the hitherto identified small GPT-binding proteins does also apply to the ryh1 protein. The human rab6 protein (Zahraoui *et al.*, 1989), shown in this report to be a functional homologue of the *S.pombe* ryh1 protein, displays a degree of sequence identity exceeding 70%. In addition, we have recently identified a gene in the budding yeast *S.cerevisiae* with a similar relatedness to the *S.pombe rhy1* gene product (L.Hengst and D.Gallwitz, unpublished).

ras, rho and ypt proteins are characterized by one or several cysteine residues near or at their C termini. Whereas ras and rho proteins typically end with a cysteine followed by three other amino acids (for review see Barbacid, 1987), ypt proteins, like ypt1 from mouse (Haubruck *et al.*, 1987; Touchot *et al.*, 1987), *S.cerevisiae* (Gallwitz *et al.*, 1983) and *S.pombe* (Miyake and Yamamoto, 1990) and several other members of this family (for review see Gallwitz *et al.*, 1989), carry two consecutive cysteine residues at their C-terminal end. In the case of the mammalian ras proteins (Willumsen *et al.*, 1984; Hancock *et al.*, 1989), the yeast Ras proteins (Fujiyama and Tamanoi, 1986; Deschenes and Broach, 1987) and the yeast Ypt1 protein (Molenaar *et al.*, 1988), it has been demonstrated that one of the C-terminal cysteines is post-translationally modified by a lipid moiety, a prerequisite for membrane attachment of the respective proteins. The *S.pombe* ryh1 protein described here, its human homologue H-rab6 and the other small GTP-binding proteins, like H-rab3A, H-rab3B and H-rab4 (Zahraoui *et al.*, 1989), have the C-terminal sequence Cys-X-Cys. This structural feature is suggestive for lipidation and membrane association of the ryh1 protein. This question has not yet been addressed experimentally.

Recent genetic, cell biological and biochemical analyses in *S.cerevisiae* provided evidence for the essential function of several ras-related proteins in distinct steps of protein transport from the ER through the different Golgi compartments to the plasma membrane. Whereas Ypt1p and Sar1p seem to play their role in ER → Golgi transport (Schmitt *et al.*, 1988; Segev *et al.*, 1988; Nakano and Muramatsu, 1989; Baker *et al.*, 1990), Sec4p is required for vesicle transport from the Golgi apparatus to the plasma membrane (Goud *et al.*, 1988; Walworth *et al.*, 1989). A reasonable assumption is that other GTP-binding proteins likewise are regulators of intracellular transport processes. The inhibition in mammalian *in vitro* systems by the non-hydrolyzable GTPγS of vesicular transport between Golgi cisternae (Melancon *et al.*, 1987) and from pre-lysosomes to the *trans*-Golgi network (Goda and Pfeffer, 1988) can be taken as an indication for this assumption. As the budding yeast *S.cerevisiae* has morphologically rather ill-defined Golgi structures, whereas in *S.pombe* typical Golgi stacks are easily observable (Johnson *et al.*, 1982; Chappel and Warren, 1989), the fission yeast is expected to allow a profitable access to examination of Golgi transport processes. The possible involvement of the newly discovered ryh1 protein in protein transport was studied by following the fate of invertase, a highly glycosylated and secreted enzyme (Moreno *et al.*, 1985), in a *ryh1* null mutant. Although derepressed invertase was not accumulated within the mutant cells, the enzyme exhibited a faster electrophoretic mobility compared to invertase of wild-type cells. Although this seems to be a minor and possibly secondary effect, we consider two, admittedly rather speculative explanations. The observation could mean that in cells lacking ryh1 protein, invertase is either underglycosylated or else is partially degraded. Mutants of *S.cerevisiae* defective in glycosylation, whose biochemical deficiencies are unclear, have been shown to be fully competent for protein secretion (for review see Ballou, 1982). In analogy to this situation, it would not be surprising to find the invertase of *ryh1*$^-$ cells outside the plasma membrane. The fact that in *ryh1*$^-$ null mutants the amount of invertase was always found to be diminished could also be taken as indication for enhanced degradation. For an explanation, one might speculate that hydrolysing enzymes destined to be transported to the vacuole are missorted to become secreted.

Since the disruption of either the *S.pombe ypt1, ypt2* or *ypt3* gene is lethal (Haubruck *et al.*, 1990; Miyake and Yamamoto, 1990) but the inactivation of the *ryh1* gene is not, the existence of a second *ryh* allele should be considered. However, neither from Southern nor from Northern analysis is there any indication for another related gene. The single *S.pombe ras1* gene, in fact, is a precedent for a gene encoding a small GTP-binding protein that is not essential for cell viability (Fukui *et al.*, 1986).

The ts phenotype of the *ryh1* null mutant provided the possibility to search for a complementing gene from other eukaryotic species. The extraordinarily high evolutionary conservation of proteins belonging to the ras superfamily could already be correlated, in a number of cases, with their specific functioning: *S.cerevisiae ras1,2* and *ypt1* null mutants are complemented by the human H-*ras* and the mouse *ypt1* (*rab1*) gene respectively (Kataoka *et al.*, 1985; DeFeo-Jones *et al.*, 1985; Haubruck *et al.*, 1989). Likewise, the *S.pombe ypt1* gene product can functionally replace the Ypt1 protein in *S.cerevisiae* (Miyake *et al.*, 1990) and the *ypt2* gene from the fission yeast complements a *S.cerevisiae sec4* mutant strain (Haubruck *et al.*, 1990). We noted that the different proteins that complement each other functionally are characterized by an identical 'effector region' which in the H-ras protein has been shown to be part of a loop and an adjacent β-strand at the surface of the protein (Pai *et al.*, 1989) and to bind a GTPase-activating protein (for review see McCormick, 1989). It seems, therefore, that the 'effector loop' is one of the structural determinants for the functional specificity of different ras and ras-related proteins (Haubruck *et al.*, 1990). It was pleasing to observe that the human rab6 protein, sharing an identical 'effector region' only with the ryh1 protein in yeast, complements the ts *ryh1*$^-$ mutant. This complementation analysis was done with the human *rab6* cDNA on a multi-copy plasmid and under transcriptional control of either the efficiently acting *S.pombe adh* or the moderately active SV40 promoter, since our previously reported results on the complementation of the essential *S.cerevisiae* Ypt1p by the mouse ypt1p had established the necessity of overproducing the mouse protein due to its relative instability in yeast (Haubruck *et al.*, 1989). We have not tested whether high expression of other GTP-binding proteins complements *ryh1*$^-$ mutants. We feel this to be rather unlikely since the different GTP binders fulfil separate and essential functions. In line with this notion is our finding that the *S.pombe* ypt2p, highly expressed from a *GAL10* promoter-controlled gene, is unable to complement *S.cerevisiae ypt1* null mutants (Haubruck *et al.*, 1990).

We would like to stress again that the ability of the human rab6p to complement *ryh1*$^-$ mutants is no definite proof for the same function of the two proteins in the respective organisms. As we have recently identified a close homologue of the *S.pombe* ryh1 and the human rab6 protein in the budding yeast *S.cerevisiae* (L.Hengst and D.Gallwitz, unpublished), the cellular function of this protein can now be studied in two evolutionarily very distant and genetically tractable eukaryotes.

Regarding the 'effector loop' of the ryh1 protein, YQATIGIDF, we noted that an intron interrupts the gene following the second codon of this structure (Figure 1). This would not be in favour of the widely held hypothesis that functional domains are encoded in uninterrupted exons. Considering the evolutionary history of genes belonging to the ras superfamily in *S.pombe*, it is also worth mentioning that the positions of introns in *ras1* (one intron), *ypt1* (four introns), *ypt3* (two introns) and *ryh1* (three introns) are in no case identical (Fukui and Kaziro, 1985; Miyake and Yamamoto, 1990; and this report).

Materials and methods

Bacterial and yeast strains

E.coli strains DH5, NM522 and RR1 were used for cloning experiments and the expression of the *ryh1* cDNA. The *S.pombe* strains used were JY741 (*h*$^-$ *ade6-M216 ura4-D18 leu1-32*) and JY746 (*h*$^+$ *ade6-M215 ura4-D18 leu1-32*). Diploids were formed by mating both strains.

Media and standard procedures

Cloning procedures and *E.coli* transformation were performed according to Maniatis *et al.* (1982) and Hanahan (1985). Yeast media have been described by Gutz *et al.* (1974) and Nurse (1975) and yeast transformation was carried out with protoplasts generated according to Beach and Nurse (1981). Standard genetic procedures described for *S.pombe* (Gutz *et al.*, 1974) were followed.

Plasmids

The *S.pombe* expression vectors pEVP11 and pSM2 were kindly provided by P.Nurse, Oxford. The plasmid pEVP11 contains a 700 bp *Eco*RI–*Sph*I fragment of the *S.pombe adh* promoter, which functions as a strong promoter in the fission yeast (Russel and Nurse, 1986). The plasmid pSM2 is a derivative of pDB248, made by inserting the SV40 early promoter. Genes linked to this promoter are expressed at moderate levels (Moreno *et al.*, 1990). Both vectors carry the *S.cerevisiae LEU2* gene for the complementation of *leu1*$^-$ *S.pombe* strains. The bacterial plasmid pT7T3-18U was obtained from Pharmacia. The pUC8-derived *E.coli* expression vector pLN has been described previously (Wagner *et al.*, 1987).

Gene disruption

One-step gene disruption was carried out according to Rothstein (1983). The construction ryh1-D1 was obtained by deletion of a 0.7 kb *Eco*RV–*Dra*I fragment and insertion of a 1.75 kb fragment containing the *ura4* gene into the *Nru*I site 8 bp 3′ of the deletion end point of the *ryh1* gene. The linear 4.2 kb *Hin*dIII fragment carrying the disrupted *ryh1* gene was isolated by the freeze-squeeze method (Tautz and Renz, 1983) and used for transformation of an *ura4*$^-$ *leu1*$^-$ diploid.

DNA and RNA procedures

All DNA manipulations were performed according to standard procedures (Maniatis *et al.*, 1982). Preparation of fission yeast chromosomal DNA has been described by Durkacz *et al.* (1986). Southern and Northern transfers to GeneScreen (NEN) or Nytran-NY13N membrane (Schleicher & Schüll) were performed using standard procedures. Hybridization was carried out at 65°C in 1 M NaCl, 10% dextran sulphate, 1% SDS and 100 μg/ml salmon sperm DNA as carrier. For isolation of *S.pombe* RNA, 2 ml of logarithmically growing cells were collected by centrifugation, washed with water and resuspended in 350 μl lysis buffer (0.1 M Tris–HCl, pH 7.5, 25 mM DTT, 40 mM LiCl). Cells were broken by vortexing with 1 g glass beads (diameter 500 μm) and 1 ml phenol for 2 min, the aqueous phase was extracted two more times with phenol and then RNA was ethanol-precipitated by adding 30 μl 4 M sodium acetate and 1 ml ethanol. All extractions to isolate RNA were carried out at 4°C.

Isolation of a ryh1 cDNA by PCR

The coding region of the *ryh1* cDNA was cloned by PCR amplification (Saiki *et al.*, 1988) from an uncloned cDNA population (kindly provided by P.Wagner, Oxted). The primers ryh1-Nde (5′-CTTTTTGTCATA-TGTCAGAAAATTACTCG-3′) and ryh1-Dra (5′-CACTGACATGTT-TAAAGAGATACACTAGC-3′) used during this reaction were designed to create a *Nde*I site at the start codon (deletion of bp −1) and a *Dra*I site in the 3′-non-coding region (A → T transversion and G → A transition in positions +9 and +12). PCR was performed using an Ericomp Programmable Reactor at 30 cycles of 95°C for 1.25 min, 48°C for 2.5 min and 70°C for 3 min each.

Expression of the rab6 cDNA in S.pombe

The *rab6* cDNA (Zahraoui *et al.*, 1989) was cloned as a 1.2 kb *Not*I–*Eco*RI fragment in the *Bam*HI (pEVP11) or the *Pvu*II (pSM2) sites of the *S.pombe* expression vectors, after generating blunt ends with Klenow enzyme. The enzyme *Not*I cuts the *rab6* cDNA at position −20 upstream of the start codon. The recombinant plasmids pADH-rab6 and pSV40-rab6 were used to transform a *ryh1*$^-$ *leu1-32* haploid strain obtained from a tetrad of a diploid with one disrupted *ryh1* allele.

Expression of the ryh1 cDNA in E.coli

For the expression of the ryh1 protein in *E.coli*, a *Nde*I–*Dra*I fragment containing the coding region of the *ryh1* cDNA was isolated. The *E.coli* expression vector pLNYPT1 (Wagner *et al.*, 1987) was cut with *Bss*HII and, after generating blunt ends, cut with *Nde*I to delete the coding region of the *S.cerevisiae YPT1* gene. This region was replaced by the *ryh1* coding region on a 618 bp *Nde*I–*Dra*I fragment. Expression of the ryh1 protein, separation of bacterially produced proteins by SDS–PAGE, electrophoretic

transfer of proteins to nitrocellulose and nucleotide binding were done as described previously (Wagner *et al.*, 1987).

Invertase assay

S.pombe invertase was isolated from cells grown at 30°C and detected according to Moreno *et al.* (1985). Briefly, protoplasts were generated by zymolase and proteins, solubilized during formation of protoplasts (external invertase) and after their lysis (intracellular invertase), were analysed electrophoretically. Electrophoresis was performed under non-denaturing conditions in 3.5% polyacrylamide slab gels for 14 h at 30 mA.

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References

Ballou,C. (1982) In Strathern,J., Jones,E. and Broach,J. (eds), *The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 335–360.

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

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

Beach,D. and Nurse,P. (1981) *Nature*, **290**, 140–142.

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.

Botstein,D., Segev,N., Stearns,T., Hoyt,M.A., Holden,J. and Kahn,R.A. (1988) *Cold Spring Harbor Symp. Quant. Biol.*, **53**, 629–636.

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

Chappel,T.G. and Warren,G. (1989) *J. Cell Biol.*, **109**, 2693–2702.

Chardin,P., Touchot,N., Zahraoui,A., Pizon,V., Lerosey,I., Olofson,B. and Tavitian,A. (1989) In Bosch,L., Kraal,B. and Parmeggiani,A. (eds), *The Guanine-nucleotide Binding Proteins.* Plenum Press, New York; NATO ASI Series A: Life Sciences, Vol. 165, pp. 153–163.

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.

Deschenes,R.J. and Broach,J.R. (1987) *Mol. Cell. Biol.*, **7**, 2344–2351.

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.

Durkacz,B., Beach,D., Hayles,J. and Nurse,P. (1986) *Mol. Gen. Genet.*, **201**, 543–545.

Fujiyama,A. and Tamanoi,F. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1266–1270.

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., Halfter,H. and Mertins,P. (1987) In Kinghorn,J.R. (ed.), *Gene Structure in Eukaryotic Microbes.* IRL Press, Oxford, pp. 27–40.

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 Parmeggiani,A. (eds), *The Guanine-nucleotide Binding Proteins.* Plenum Press, New York; NATO ASI Series A: Life Sciences, Vol. 165, pp. 257–264.

Gilman,A.G. (1987) *Annu. Rev. Biochem.*, **56**, 615–649.

Goda,Y. and Pfeffer,S.R. (1988) *Cell*, **55**, 309–320.

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.

Hanahan,D. (1985) In Glover,D.M. (ed.), *DNA Cloning: A Practical Approach.* IRL Press, Oxford, Vol. 1, pp. 109–135.

Hancock,J.F., Magee,A.I., Childs,J.E. and Marshall,C.J. (1989) *Cell*, **57**, 1167–1177.

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.

Haubruck,H., Engelke,U., Mertins,P. and Gallwitz,D. (1990) *EMBO J.*, **9**, 1957–1962.

Johnson,B.F., Calleja,G.B., Yoo,B.Y., Zuker,M. and McDonald,I.J. (1982) *Int. Rev. Cytol.*, **75**, 167–208.

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

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

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Matsui,Y., Kikuchi,A., Kondo,J., Hishida,T., Teranishi,Y. and Takai,Y. (1988) *J. Biol. Chem.*, **263**, 11071–11074.

McCormick,F. (1989) *Cell*, **56**, 5–8.

Melancon,P., Glick,B.S., Malhotra,V., Weidman,P.J., Serafini,T., Gleason,M.L., Orci,L. and Rothman,J.E. (1987) *Cell*, **51**, 1053–1062.

Mertins,P. and Gallwitz,D. (1987) *EMBO J.*, **6**, 1757–1763.

Miyake,S. and Yamamoto,M. (1990) *EMBO J.*, **9**, 1417–1422.

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

Moreno,S., Ruíz,T., Sánchez,Y., Villanueva,J.R. and Rodríguez,L. (1985) *Arch. Microbiol.*, **142**, 370–374.

Moreno,S., Klar,A. and Nurse,P. (1990) *Methods Enzymol.*, in press.

Nakano,A. and Muramatsu,M. (1989) *J. Cell Biol.*, **109**, 2677–2691.

Nurse,P. (1975) *Nature*, **256**, 547–551.

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

Rothstein,R.J. (1983) *Methods Enzymol.*, **101**, 202–211.

Russel,P. and Nurse,P. (1986) *Cell*, **45**, 145–153.

Saiki,R.K., Gelfand,D.H., Stoffel,F., Scharf,S.J., Higushi,R., Horn,G.T., Mullis,K.B. and Erlich,H.A. (1988) *Science*, **239**, 487–491.

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

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.

Sewell,J.L. and Kahn,R.A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4620–4624.

Tautz,D. and Renz,M. (1983) *Anal. Biochem.*, **132**, 14–19.

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

Wagner,P., Molenaar,C.M.T., Rauh,A.J.G., Brökel,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.

Wigler,M., Field,J., Powers,S., Broek,D., Toda,T., Cameron,S., Nikawa,J., Michaeli,T., Colicelli,J. and Ferguson,K. (1988) *Cold Spring Harbor Symp. Quant. Biol.*, **53**, 649–655.

Willumsen,B.M., Norris,K., Papageorge,A.G., Hubbert,N.L. and Lowy,D.R. (1984) *EMBO J.*, **3**, 2581–2585.

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

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