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Schizosaccharomyces pombe ypt5: A Homologue of the rab5 Endosome Fusion Regulator

John Armstrong,*† Mark William Craighead,‡ Rose Watson,‡ Sreenivasan Ponnambalam,‡ and Sally Bowden‡

*School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, England; and ‡Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, England

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The ypt/rab proteins are a family of small GTP-binding proteins thought to be required for different stages of membrane traffic. From the fission yeast *Schizosaccharomyces pombe* we have isolated and characterized *ypt5*, a gene encoding a homologue of rab5, a mammalian protein apparently involved in regulating fusion of early endosomes. Recombinant ypt5 protein bound GTP. The *ypt5* gene was found to be essential for viability on minimal media, but *ypt5*-disrupted cells grew slowly on some rich media and accumulated a population of small vesicles not observed in wild-type cells. Canine rab5 cDNA could replace the *ypt5* gene in *S. pombe* and restore normal growth and viability. Ypt5 protein expressed in mammalian cells colocalized with the transferrin receptor to early endosomes. Thus, molecular aspects of the early endocytic pathway may be conserved between mammalian cells and *S. pombe* and hence may be amenable to genetic analysis.

INTRODUCTION

In eukaryotic cells the basic processes of secretion and endocytosis are thought to be mediated by vesicles that carry out transport of materials between different membrane compartments. The molecular analysis of vesicle formation, targeting, and specificity are currently the focus of much study. A family of small GTP-binding proteins related to the p21-ras oncogene product, the ypt or rab proteins, appear to play important roles in these processes. The first reported members of the family, Ypt1p and Sec4p from the budding yeast *Saccharomyces cerevisiae*, are involved in transport from the endoplasmic reticulum (ER) to and through the Golgi and from the Golgi to the bud plasma membrane, respectively (Salminen and Novick, 1987; Segev *et al.*, 1988). From these observations arose the hypothesis that a different member of the ypt family might be required to target vesicles from each step of the secretory pathway (Bourne, 1988). Subsequent studies of Sec4 protein have indicated a cyclic mechanism of association with vesicles, targeting to the destination membrane and release into the cytosol for a subsequent round of function, coupled to a regulated cycle of binding, hydrolysis, and release of nucleotide (Walworth *et al.*, 1989; Sasaki *et al.*, 1991). Consistent with their proposed function, ~20 distinct members of the ypt/rab family, as well as several minor variants, have been reported from mammalian cells (Haubruck *et al.*, 1987; Touchot *et al.*, 1987; Zahraoui *et al.*, 1989; Chavrier *et al.*, 1990b, 1992; Elferink *et al.*, 1992). Some of the proteins have been localized to different compartments involved in constitutive and regulated secretion (Chavrier *et al.*, 1990a; Fischer von Mollard *et al.*, 1990; Goud *et al.*, 1990; Plutner *et al.*, 1991). In addition, rab4, rab5, and rab7 are found in endosomal structures (Chavrier *et al.*, 1990a; Van der Sluijs *et al.*, 1991), implicitly extending the hypothesis to include endocytosis. In support of this, evidence has been presented that rab5 is involved in fusion of early endosomes in vitro (Gorvel *et al.*, 1991) and in vivo (Bucci *et al.*, 1992), whereas rab4 appears to play a part in return of vesicles from the endosome to the cell surface (Van der Sluijs *et al.*, 1992). Recently, a homologue of rab7 from *S. cerevisae* has been described (Wichmann *et al.*, 1992); disruption of the gene perturbed the transport of various markers to the vacuoles, a step analogous to the delivery of endocytosed material to the lysosomes of higher cells.

We are investigating the role of the ypt proteins in the fission yeast *Schizosaccharomyces pombe*. This organism, like *S. cerevisiae*, is amenable to genetic analysis but differs radically from it and in some respects may

† Corresponding author.

be a closer model of higher cells. This is particularly true of the control of its cell cycle (Lee and Nurse, 1987). With respect to membrane traffic, the ER of *S. pombe* contains a distinct reticular structure reminiscent of the peripheral reticulum of higher cells (Pidoux and Armstrong, 1992). The Golgi complex of fission yeast is readily detectable by conventional methods as a stack of flattened cisternae (Smith and Svoboda, 1972). As with *S. cerevisiae*, glycoproteins are modified with polymannose structures but in addition acquire terminal galactose (Moreno *et al.*, 1985; Chappell and Warren, 1989).

We and others have previously reported the isolation of three ypt genes from *S. pombe*. Ypt1p is slightly closer in sequence to its mammalian homologue, rab1, than to Ypt1p of *S. cerevisiae* (Fawell *et al.*, 1989; Miyake and Yamamoto, 1990). Ypt2p has some homology to Sec4p and can complement a mutation in this gene (Fawell *et al.*, 1990; Haubruck *et al.*, 1990), but it is closer in sequence to mammalian rab8. The functions of ypt3p (Fawell *et al.*, 1990; Miyake and Yamamoto, 1990) and its mammalian homologue rab11 (Chavrier *et al.*, 1990b) are as yet unknown. In addition, *ryh1*, whose product is homologous to rab6, has been identified (Hengst *et al.*, 1990).

We describe here the isolation of a further gene from *S. pombe*, *ypt5*. The encoded protein has extensive sequence homology to mammalian rab5. We present genetic and biochemical analyses of the *ypt5* gene, its product, and its relationship to rab5. The effects of gene disruption on growth and morphology have been examined, and the localization of ypt5 protein expressed in mammalian cells has been determined. We discuss the possibility of conservation of function between ypt5 protein and rab5.

MATERIALS AND METHODS

S. pombe Strains

Strains used for transformation were 556 (h^+ *ade6*-M216 *ura4*-D18 *leu1*-32) and 611 (h^+/h^- *ade6*-M216/*ade6*-M210 *ura4*-D18/*ura4*-D18 *leu1*-32/*leu1*-32), both from P. Nurse (University of Oxford, Oxford, UK).

Cloning and Sequence Analysis

Standard procedures (Sambrook *et al.*, 1989) were used for manipulation, analysis, and subcloning of phage and plasmid DNA. DNA sequences were determined using Sequenase (United States Biochemical, Cleveland, OH) by chain-terminator sequencing (Tabor and Richardson, 1987).

A library of *S. pombe* genomic DNA in the vector λ-dash was screened with the mixed oligonucleotides GATACKGCKGGKCAGGAGCG and CGTTCTTGKCCKGCKGTGTC (Touchot *et al.*, 1987) as previously described (Fawell *et al.*, 1990, 1992). From one clone a hybridizing 3.3-kilobase (kb) *Eco*RI restriction fragment was subcloned into Bluescript (Promega, Madison, WI) to generate the plasmid pYPT5. Further subclonings and sequencing of overlapping fragments were used to give the sequence of the ypt5 gene; this also revealed that the 3′ *Eco*RI site derived from the phage vector rather than the *S. pombe* genome. From an independent phage clone, an overlapping 1.65-kb *Xba* I fragment was subcloned (Figure 4A) to extend the sequence in the 3′ direction.

Expression of ypt5 Protein in Escherichia coli and Analysis of GTP Binding

From total *S. pombe* RNA, ypt5 cDNA with terminal *Bam*HI sites was obtained by polymerase chain reaction (PCR) (Newman *et al.*, 1992). The product was subcloned and sequenced to confirm the absence of mutations and then inserted into the *Bam*HI site of pGEX2T (Smith and Johnson, 1988). The resulting plasmid encoded an in-frame fusion of glutathione-S-transferase to ypt5, separated by a thrombin cleavage site. Fusion protein was affinity-purified from extracts of *E. coli* using glutathione-agarose essentially as described (Smith and Johnson, 1988). Typically, 1 l of culture yielded 3–5 mg of fusion protein, which was >95% pure as judged by sodium dodecyl sulfate (SDS) gel electrophoresis. Purified fusion protein was subjected to SDS gel electrophoresis, transferred electrophoretically to nitrocellulose, and tested for the ability to bind [α-^{32}P]GTP (Lapetina and Reep, 1987). Alternatively, fusion protein was first cleaved with thrombin (Sigma, St. Louis, MO) (Smith and Johnson, 1988) and the products analyzed as before.

Yeast Genetics and Growth

Media and basic procedures for growth and sporulation of *S. pombe* were as described (Moreno *et al.*, 1990; Armstrong *et al.*, 1992). Yeast extract (YE) medium contains 5 g/l yeast extract (Difco, Detroit, MI); 30 g/l glucose; 100 μg/ml each adenine, leucine, and uracil; and vitamin and mineral mixtures as for minimal media. Transformation of *S. pombe* with both linear DNA and plasmids was by the lithium chloride method (Broker, 1987).

Disruption of the ypt5 Gene

A plasmid was constructed in which a 1.7-kb *Hin*dIII fragment, including the *S. pombe ura4* gene (Grimm *et al.*, 1988), was inserted into the *Nde* I site of pYPT5 (base 916 in Figure 1) to generate pYPT5-ura (Figure 4A). From this, the ypt5 gene and flanking regions, interrupted by the ura4 gene, were excised using *Sph* I and *Sst* I, gel-purified, and used to transform the diploid *S. pombe* strain 611. Ura^+ transformants were screened for homologous integration by PCR. A loopful of cells was incubated in 20 μl 1% (vol/vol) Triton X-100 (Sigma, St. Louis, MO), 5 mM tris(hydroxymethyl) aminomethane-HCl, pH 7.6, and 0.1 mM EDTA at 95° for 5 min. From this, 1 μl was added to a 20 μl PCR as described (Pidoux and Armstrong, 1992) using a 5′ primer from near the 3′ end of the ura4 gene and a 3′ primer from 150-base pairs (bp) beyond the 3′ end of the region used for transformation. Transformants yielding the predicted 750-bp product were further analyzed by Southern blotting of genomic DNA (Pidoux and Armstrong, 1992) using as probe a *Spe* I-*Eco*RV fragment adjacent to the region used for transformation (Figure 4A). Heterozygous diploid cells were sporulated and the resulting tetrads dissected. Spores were germinated on YE medium and replicated onto various rich and minimal media.

Replacement of the ypt5 Coding Sequence with rab5 cDNA

The ypt5 coding sequence (nucleotides 25–1113 of Figure 1) was precisely replaced with canine rab5 cDNA by a combination of PCR and subcloning. The rab5 sequence (Chavrier *et al.*, 1990a) was amplified from a plasmid (from M. Zerial, EMBL, Heidelberg) using primers that introduced, respectively, a *Nco* I site at the start codon and a *Bam*HI site after the stop codon. The 5′ upstream region of the ypt5 gene was amplified from pYPT5 using as primers a plasmid sequence and the complement of nucleotides 2–31 (Figure 1) altered at positions 22 and 23 to introduce a *Nco* I site. Both products were digested with *Nco* I, ligated, and reamplified with the two outer primers. The product

```
1
CTTGTTGTTACACTCAAAATAAATATGGCATCAAATACAGCTCCAAAGAATGTCGTTACAATCAATCAGAAGCTTGTTCT
                        M  A  S  N  T  A  P  K  N  V  V  T  I  N  Q  K  L  V  L
81
TCTTGGTGATTCCGCCGTTGGCAAAgtaggTCATTCATTCAAGAACTAATTTTATATATTTTGTCGTTTTAGAGctaatT
 L  G  D  S  A  V  G  K  [
161
GTTTATAATAATTtagAGTAGTCTCGTTCTTCGATTCGTTAAAGATCAATTTGATGgtaagAATTTACTTATGAGGCTTT
              ]S  S  L  V  L  R  F  V  K  D  Q  F  D  D[
241
TAATTTTTCATGCAATAGAAAActgacATTTTTCCTCTAAAcagATTACAGAGAAAGCACCATTGGAGgtatgCAAGCAC
                                          ]  Y  R  E  S  T  I  G  A[
321
AATAATGGGAAAGGTTTCTCTCCTTTTAATCGTATCATTTTTTctgacTTTCAAACTTGTTcagCTGCTTTTTTGACTCA
                                                                  ]  A  F  L  T  Q
401
AACCCTTCCTATAGATGAAAACACTTCCGTAAAACTCGAAATTTGgtatgGAAGTTTAATGCAGCAAAATTTATTAATTT
 T  L  P  I  D  E  N  T  S  V  K  L  E  I  W  [
481
GTTTTATTTTTCAActaacTAAATTTtagGGATACGGCTGGTCAGGAACGTTATAAATCTCTAGCTCCTATGTACTgtat
                           ]  D  T  A  G  Q  E  R  Y  K  S  L  A  P  M  Y  Y[
561
gTTGTAGATGTGCCGCTTTATTTAttaacTTGGTtagATCGAAATGCTAATTGCGCTATTGTCGTATACGATATTACTCA
                                    ]  R  N  A  N  C  A  I  V  V  Y  D  I  T  Q
641
AGCTgtaagTAAAACCTCCATCATACGATATGGAAATAAGATTATGTTCGACGTTTTCTTGTTCGGTTTttaacAATATT
 A  [
721
tagGCATCTCTAGAAAAGGCCAAATCTTGGATTAAGGAACTTCAGCGTCAAGCTCCAGAGGGTATTGTTATAGCTTTGGC
  ]A  S  L  E  K  A  K  S  W  I  K  E  L  Q  R  Q  A  P  E  G  I  V  I  A  L  A
801
CGGCAACAAGCTGGATCTTGCCCAAGAACGTAGAGCTGTTGAGAAAGCTgtaagTTTTTTTTAAGTTATATACGTCTTG
 G  N  K  L  D  L  A  Q  E  R  R  A  V  E  K  A  [
881
TAGGTGTAttaacTTTTCTCtagGATGCTGAAGCATATGCCGCTGAGGCTAATCTTTTATTCTTTGAAACTTCTGCTAAG
                        ]D  A  E  A  Y  A  A  E  A  N  L  L  F  F  E  T  S  A  K
961
ACTGCTGAAAATGTAAATGAGCTTTTTACTGCTATTGCCAAAAAGCTTCCCTTAGAAGATAAGCTCAACCAGGCCAGAGG
T  A  E  N  V  N  E  L  F  T  A  I  A  K  K  L  P  L  E  D  K  L  N  Q  A  R  G
1041
CGCTGTGAATCGTGGTGTCAATTTGTCAGAAGCTCGGCCTGCTGCTCAACCAAGCGGTTCATGCTCTTGCTAATTTTTCA
 A  V  N  R  G  V  N  L  S  E  A  R  P  A  A  Q  P  S  G  S  C  S  C  *
1121
TGTTTACCTGATAATGTTGAAAATTAATGGTTCTTTGAATA
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Figure 1. Sequence of the *S. pombe ypt5* gene and encoded protein. Introns are marked by brackets; *S. pombe* splicing signals are in small letters. The sequence has been submitted to the EMBL Nucleotide Database with the accession number Z22220.

of 2.25 kb was digested with *Sph* I and *Bam*HI and then used to replace the ypt5 gene and upstream region of pYPT5-ura, generating pYPT5-rab5-ura (Figure 4A). Plasmid sequences were removed with *Sph* I and *Sst* I as before and the purified fragment used to transform *S. pombe* 556. Ura$^+$ transformants were screened by PCR and Southern blotting exactly as before. The rab5 sequence was reamplified from transformants and sequenced to confirm the absence of mutations.

S. pombe 556 and rab5 replacement strains were analyzed by Western blotting for the presence of ypt5 and rab5 proteins. Spheroplast extracts were separated by SDS gel electrophoresis, transferred to nitrocellulose, and treated with antibodies that were detected by enhanced chemiluminescence (ECL system, Amersham, Arlington Heights, VA) as described (Pidoux and Armstrong, 1992). First antibodies were rabbit antiserum to a peptide from residues 179–197 (Figure 2) conjugated and immunized as previously described for ypt1 peptides (Newman *et al.*, 1992), used at a dilution of 1:10 000 or affinity-purified rabbit anti-rab5 (Chavrier *et al.*, 1990a) (from M. Zerial, EMBL, Heidelberg) at a dilution of 1:20 000.

```
rab5a     1 MAnrgaTrPngpNtgnkIcQfKLVLLGeSAVGKSSLVLRFVKgQFhefqESTIGAAFLTQT
             ||    | |   |    | | |||||| ||||||||||||||| ||    ||||||||||||
spypt5    1 MA  snTaP  kN vvtInQ KLVLLGdSAVGKSSLVLRFVKdQFddyrESTIGAAFLTQT
                          ---B1---  L1  --A1---      L2       ---B2--

rab5a    62 vclD dTtVKfEIWDTAGQERYhSLAPMYYRgAqaAIVVYDITneeSfarAKnWvKELQRQ
               | | || ||||||||||| |||||||| | ||||||||   |   || | |||||||
spypt5   56 lpiDenTsVKlEIWDTAGQERYkSLAPMYYRnAncAIVVYDITqaaSlekAKsWiKELQRQ
            -- L3 ----B3----  L4   ---A2---- L5--B4-- L6-------A3--------

rab5a   122 AspnIVIALsGNKaDLA nkRAVdfqeAqsYAddnsLLFmETSAKTsmNVNEiFmAIAKKL
              |   ||||| ||| |||   |||    |  ||    ||| ||||||  |||| | ||||||
spypt5  117 ApegIVIALaGNKlDLAqerRAVekadAeaYAaeanLLFfETSAKTaeNVNElFtAIAKKL
              L7  --B5--     L8      ----A4---- L9 -B6   L10  -----A5-----

rab5a   182 PknEpqnpgAnsArgRGVdLtE ptqptrSqcCSn.
            | |    |  | ||| | |      | || |
spypt5  178 P lEdklnqArgAvnRGVnLsEarpaaqpSgsCSc.
```

Figure 2. Comparison of the amino acid sequences of *S. pombe* ypt5 and canine rab5a proteins (Chavrier *et al.*, 1990a). Secondary structures of the homologous regions of p21ras are shown for α-helix (A), β-sheet (B), and loops (L).

Microscopy

Log-phase cultures of *S. pombe* were photographed on a Zeiss Axiophot microscope (Thornwood, NY) using Nomarski optics and a 100× objective.

Electron microscopy of permanganate-fixed *S. pombe* was performed as described (Chappell and Warren, 1989). *S. pombe* cells were pelleted and washed three times in sterile water and then fixed for 40 min in 2% (wt/vol) potassium permanganate at room temperature. Cells were washed three times in sterile water, resuspended in 70% ethanol, and incubated overnight at 4°C. The samples were dehydrated by sequential washes in 70% ethanol (twice for 15 min), 90% ethanol (twice

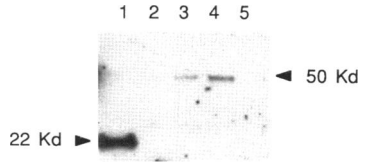

Figure 3. GTP binding of bacterially expressed ypt5 protein. Uncleaved glutathione-S-transferase-ypt5 fusion protein (lanes 2–4; 0.4, 1, 2 μg, respectively), thrombin-cleaved fusion protein (lane 1; 1 μg), and glutathione-S-transferase (lane 5; 2 μg) were separated by SDS gel electrophoresis, electroblotted onto nitrocellulose, and incubated with [α-^{32}P]GTP as described in MATERIALS AND METHODS.

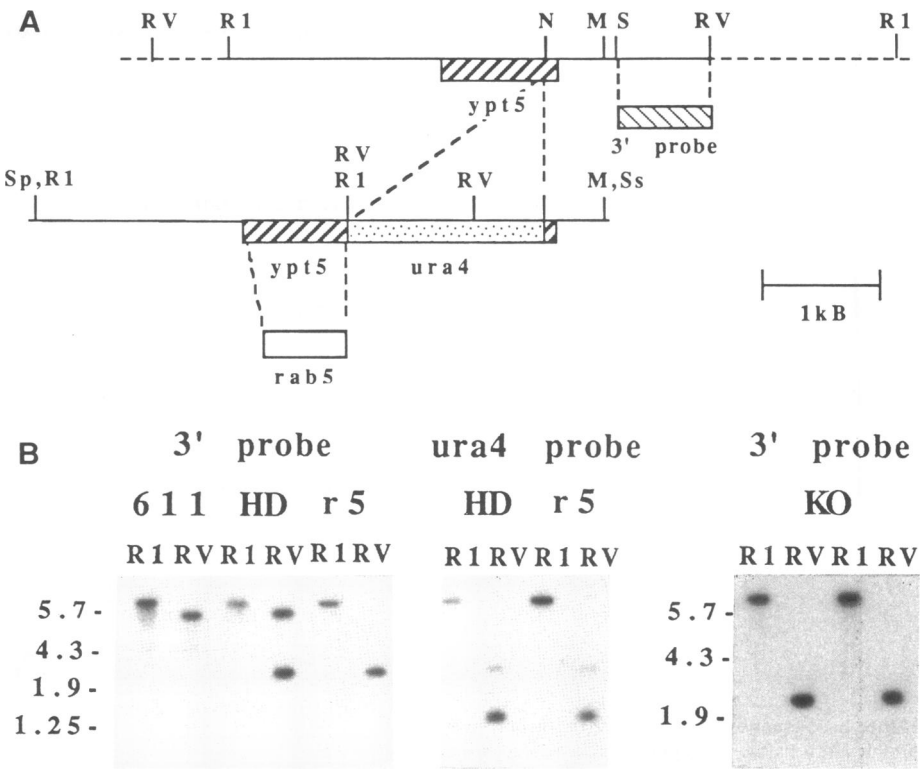

Figure 4. (A) Structures of the *S. pombe ypt5* gene and derived plasmids and probes. Selected restriction sites are marked: M, *Mbo* I; N, *Nde* I; RI, *Eco*RI; RV, *Eco*RV; S, *Spe* I; Sp, *Sph* I; Ss, *Sst* I. The *S. pombe ura4* gene and canine rab5 cDNA were inserted as shown (see MATERIALS AND METHODS). Regions shown as dotted have not been cloned. (B) Southern blotting analysis of *S. pombe* diploid strain 611 (611), heterozygous diploid with one copy of the *ypt5* gene disrupted by *ura4* (HD), haploid cells in which the ypt5 coding region has been replaced by rab5 cDNA (r5), and two haploid disruptants from a heterozygous diploid tetrad (KO). DNA was restricted with *Eco*RI (RI) or *Eco*RV (RV). Blots were probed with a fragment from the 3′ side of the *ypt5* gene beyond the region used for transformation (A) or the *ura4* gene (which is deleted from the parental strains 611 and 556). Approximate mobilities of marker fragments of known sizes (in kb) are shown. The two large *Eco*RI fragments from HD that hybridize to the 3′ probe (see A) are not resolved.

for 15 min), and 100% ethanol (three times for 20 min) at room temperature. The pellet was resuspended in propylene oxide for 10 min and then incubated in a 1:1 mixture of propylene oxide and Epon resin (Taab Laboratories, Reading, UK) for 1 h and neat Epon resin twice for 2 h. The sample was then embedded in Epon resin at 65°C overnight. Blocks were sectioned with a diamond knife, stained with uranyl acetate and lead citrate, and examined in a Philips CM10 electron microscope (Eindhoven, Netherlands).

Expression of ypt5 in HeLa Cells

Ypt5 cDNA was inserted into the expression vector pCMUIV (Nilsson *et al.*, 1989) and introduced into semiconfluent HeLa cells by calcium phosphate coprecipitation (Nilsson *et al.*, 1989). Cells were incubated for 16 h in the presence of the precipitate, washed, and incubated in fresh medium for a further 24 h. Paraformaldehyde fixation, permeabilization in 0.2% (vol/vol) Triton X-100, antibody labeling, and fluorescence microscopy were all as described (Armstrong *et al.*, 1989). First antibodies were rabbit anti-ypt5 peptide (see above) used at 1:400 and mouse monoclonal OKT9 anti-transferrin receptor (Sutherland *et al.*, 1981) (from T. Nilsson, Imperial Cancer Research Fund, London, England) used at 1:50. Second antibodies were fluorescein-conjugated goat anti-rabbit and rhodamine-conjugated goat anti-mouse (both Tago, Burlinghame, CA) used at 1:100.

RESULTS

Sequence of ypt5

The isolation of a group of ras-related genes from *S. pombe*, using mixed oligonucleotides encoding part of the GTP-binding site, has been described previously (Fawell *et al.*, 1990, 1992). A further member of this group, isolated in five separate genomic clones, encoded a new potential member of the ypt family. Using the consensus sequences for intron junctions in *S. pombe* (Mertins and Gallwitz, 1987), a sequence encoding a protein similar in length and characteristics to the ypt/rab family, interrupted by seven introns, was deduced (Figure 1). The coding sequence and positions of the introns were confirmed by analysis of the corresponding cDNA recovered by PCR.

The encoded protein is clearly homologous to mammalian rab5 (Figure 2), and the gene was therefore named ypt5. The overall amino acid identity is 63%, with most of the differences concentrated in the C-terminal region. This pattern of sequence conservation is similar to that observed for ypt's 1, 2, and 3 of *S. pombe* compared with their respective homologues rab1, rab8, and rab11 (Fawell *et al.*, 1989, 1990; Chavrier *et al.*, 1990b; Miyake and Yamamoto, 1990).

Binding of GTP by ypt5 Protein

To confirm that ypt5 is a GTP-binding protein, it was expressed in *E. coli* as a fusion to glutathione-S-transferase. The resulting 50-kDa fusion protein was isolated using glutathione-agarose, and the 22-kDa ypt5 portion was released by cleavage with thrombin. The fusion protein and the cleaved products were then tested for their ability to bind GTP on nitrocellulose filters. Both the fusion protein and the isolated ypt5 protein, but not the glutathione-S-transferase portion, bound GTP (Figure 3), the cleaved protein doing so more efficiently than the fusion protein.

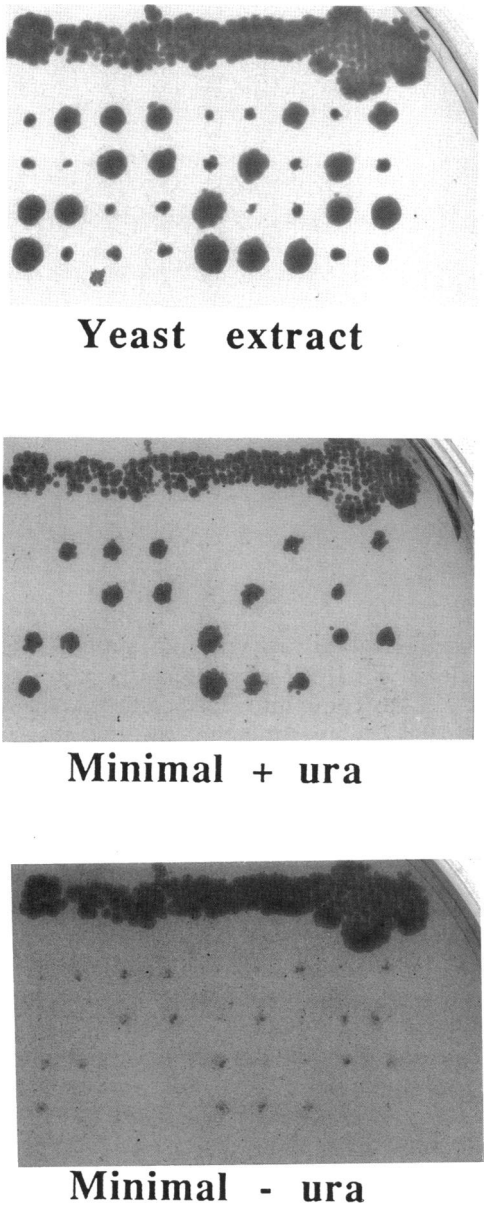

Figure 5. Tetrad analysis of heterozygous diploid *S. pombe* carrying *ura4*-disrupted *ypt5*. Nine asci from a sporulating culture were dissected, the spores germinated on YE medium and replicated to minimal medium with or without uracil. From each tetrad two progeny grew normally and were ura$^-$, and two grew slowly on YE but not on minimal media.

Gene Disruption of ypt5

To test whether ypt5 was essential for viability, a plasmid was constructed in which the ypt5 gene was interrupted by the selectable *ura4* gene (Figure 4A). The linear fragment was recovered from the plasmid and introduced into *ura*$^-$ diploid *S. pombe* cells. Resulting ura$^+$ colonies were screened by PCR for homologous integrants, and the identities of three such transformants were confirmed by Southern blotting (Figure 4B). Each of these was sporulated and analyzed by tetrad analysis. Initially, spores were germinated on YPD medium, which indicated that disruption of *ypt5* was lethal. However, germination on YE medium resulted in two normal and two slow-growing colonies from a minimum of 10 tetrads from each diploid. Replica plating and PCR showed that the normally growing progeny were all *ura*$^-$, whereas the slow-growing progeny were disruptants (Figure 5). This was confirmed by Southern blotting (Figure 4B).

Analysis of ypt5 Disruptants

The growth properties of *ypt5* disruptants were further analyzed. The disruptants failed to grow on YPD or any minimal medium tested. In liquid YE medium the cells had a doubling time of ~8 h, in contrast to 2 h 15 min for wild-type cells.

Viewed by Nomarski optics, *ypt5*-disrupted cells were smaller than normal and showed gross alterations in morphology (Figure 6). Disruptants of ypt5 were also examined by electron microscopy (Figure 7). In comparison to wild-type cells, disruptants had acquired a population of heavily staining vesicles. Occasionally, these membrane structures appeared quite asymmetric (Figure 7C), suggesting a more elaborate morphology than a simple vesicle.

Replacement of ypt5 Gene by Canine rab5 cDNA

Given the extensive sequence conservation between ypt5 and rab5 proteins, we tested the ability of the rab5 sequence to replace the *ypt5* gene. A plasmid was constructed comprising canine rab5 cDNA and the selectable *ura4* gene between flanking sequences of ypt5 (Figure 4A) to direct the precise replacement of the ypt5 coding sequence with rab5. This linear fragment was introduced into *S. pombe*, and ura$^+$ transformants were screened as before for homologous integration. Approximately 1 in 10 showed the expected amplification product; one of these was analyzed by Southern blotting to confirm that the ypt5 sequence had been replaced by rab5 (Figure 4B). Western blotting with anti-ypt5 and anti-rab5 antibodies showed that a protein of the expected size for ypt5 was present in parental cells but absent in the transformant; however, a new protein reactive with anti-rab5 antibody was present (Figure 8). The transformants showed no detectable differences from wild-type cells in growth or morphology, in rich or minimal media, and at a variety of temperatures and salt concentrations. Thus, canine rab5 is capable of substituting for ypt5 function in vivo.

Localization of ypt5 Protein in Mammalian Cells

As a further comparison of ypt5 and rab5 proteins, we investigated the localization of ypt5 protein expressed

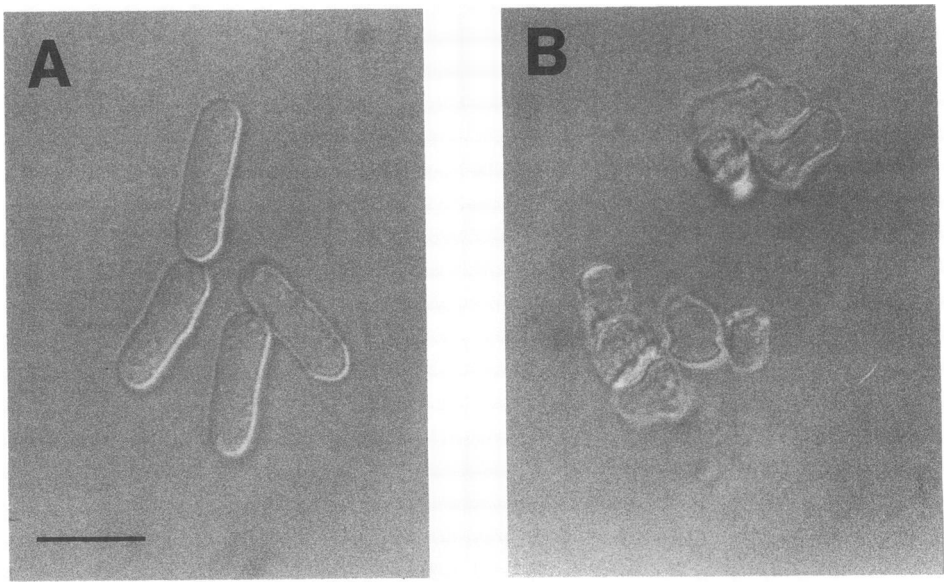

Figure 6. Microscopy of wild-type (A) and *ypt5*-disrupted (B) *S. pombe*. Cells were visualized by Nomarski optics. Bar, 10 μm.

in mammalian cells. Ypt5 protein was expressed in HeLa cells by transfection and visualized by immunofluorescence. The protein showed a punctate, presumably vesicular, distribution (Figure 9A). Labeling of transfected cells with an antibody to the transferrin receptor, a marker of the early endosome membrane, showed substantial colocalization of ypt5 protein and transferrin receptor (Figure 9). In some cells expressing high levels of ypt5 (e.g., the two lower cells of Figure 9), both ypt5 protein and transferrin receptor appeared to concentrate in enlarged structures at the tips of cell processes.

DISCUSSION

We have cloned and characterized a gene, *ypt5*, encoding a further member of the ypt-rab family of small GTP-binding proteins, from the fission yeast *S. pombe*. The protein is homologous to rab5, a mammalian protein apparently involved in directing fusion of early endosomes (Gorvel *et al.*, 1991; Bucci *et al.*, 1992). If the proteins are also homologous in function, the *ypt5* gene may provide a starting point for the analysis of the early stages of endocytosis by genetic methods in *S. pombe*. We have therefore explored the similarity of the two proteins in various ways.

The predicted amino acid sequences of ypt5 and rab5 proteins show 63% identity after introducing gaps as shown (Figure 2). This is comparable with the level of sequence conservation in other members of the ypt/rab family between *S. pombe* and higher cells. When the known secondary structure of mammalian p21-ras is superimposed (Wittinghofer and Pai, 1991), the sequence differences are seen to concentrate in predicted loop regions (Figure 2). Two unusual features of the ypt5 sequence are the additional residues in the regions of loops 3 and 8. The lengths of these regions are highly conserved throughout the ypt/rab family (cf. Figure 3 of Chavrier *et al.*, 1990b); however, the corresponding loops of p21-ras come into close contact (Wittinghofer and Pai, 1991), suggesting that the two insertions in ypt5 are structurally interdependent. As with other members of the family, the homologues diverge toward the C-terminus, suggesting that this region is less important for function (but see below). We have previously reported that the terminal structure -CSC is modified in vivo with both methyl and geranylgeranyl groups (Newman *et al.*, 1992); a similar structure in mammalian rab3a/smgp25a is similarly modified with geranylgeranyl groups on both cysteine side chains (Farnsworth *et al.*, 1991).

To begin the genetic analysis of *ypt5*, we tested the effect of disrupting the gene. This showed *ypt5* to be important for normal growth (Figure 5). Disruptant cells grew very slowly on some rich media with grossly altered morphology (Figure 6) and failed to grow on minimal medium. The disrupted gene encodes a ypt5 protein lacking one of the conserved regions involved in GTP binding and the terminal cysteine residues required for membrane attachment, fused to irrelevant sequence form the noncoding part of the *ura4* gene; thus, it is formally possible, if unlikely, that the residual protein is required for survival of the cells and that a gene deletion would be completely lethal. Whether or not this is the case, the availability of such a conditionally lethal mutant should be of use in studying the function of *ypt5* and in the isolation of suppressor genes. Examination of the disruptant cells revealed the appearance of a population of vesicles that were heavily stained after permanganate fixation (Figure 7, B and C). Similar vesicles were observed after overexpression of a mutant ypt5 protein, $ypt5^{I128}$ (Ponnambalam, Watson, and Armstrong, unpublished data); the corresponding mu-

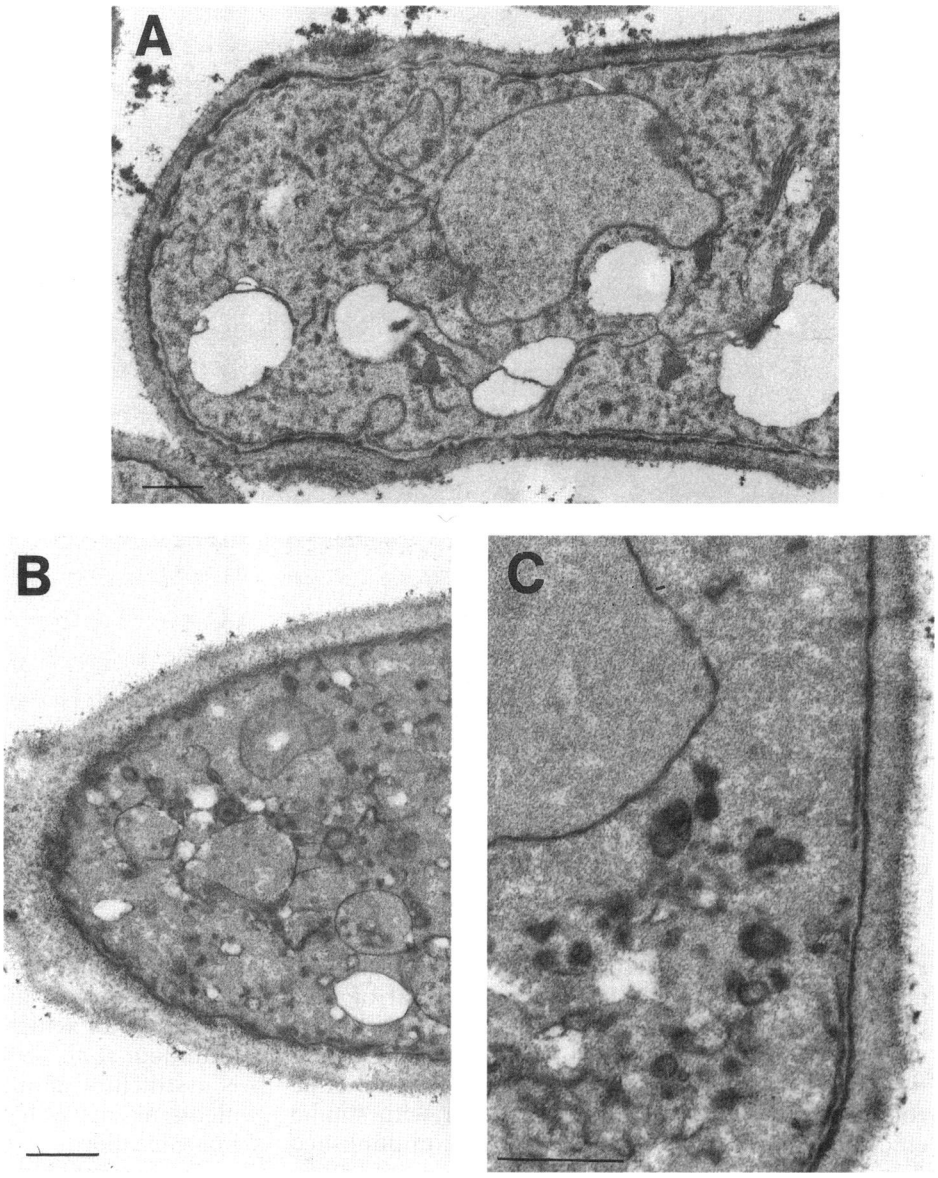

Figure 7. Electron microscopy of permanganate-fixed wild-type (A) or *ypt5*-disrupted (B and C) *S. pombe* cells. Heavily stained vesicles are apparent in the disruptant cells; some asymmetric membrane structures are also present. Bar, 0.5 μm.

tation in *ypt1* of *S. cerevisiae* confers a dominant-lethal phenotype (Schmitt *et al.*, 1986). In comparison with the vesicles seen to accumulate in secretion-defective mutants of *S. cerevisiae* (Kaiser and Schekman, 1990), these were generally larger (200–300 nm in diameter) and more heterogeneous in morphology. An intriguing possibility is that the membrane structures are accumulated intermediates of an endocytic pathway functionally related to the endocytic vesicles of higher cells; however, testing this speculation must await the development of appropriate markers for endocytosis in *S. pombe*.

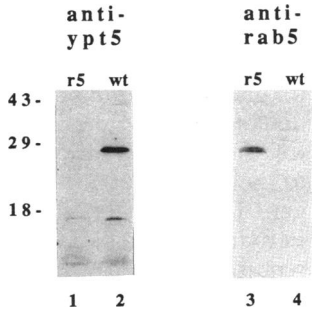

Figure 8. Detection of ypt5 and rab5 proteins in *S. pombe*. Parental strain 556 (wt) or a rab5 replacement strain (r5) (see Figure 4A) were analyzed by Western blotting, using antiserum to ypt5 peptide (anti-ypt5) or rab5 (anti-rab5). Mobilities of marker proteins, with molecular weights in kilodaltons, are shown on the left.

To investigate the functional relationship between ypt5 protein and rab5, we tested the ability of rab5 cDNA to substitute for *ypt5* and found that it could do so with no detectable effects on the cell (Figures 4 and 8). The rab5 cDNA lacks introns, has mammalian codon preferences, and is present as only a single copy at the

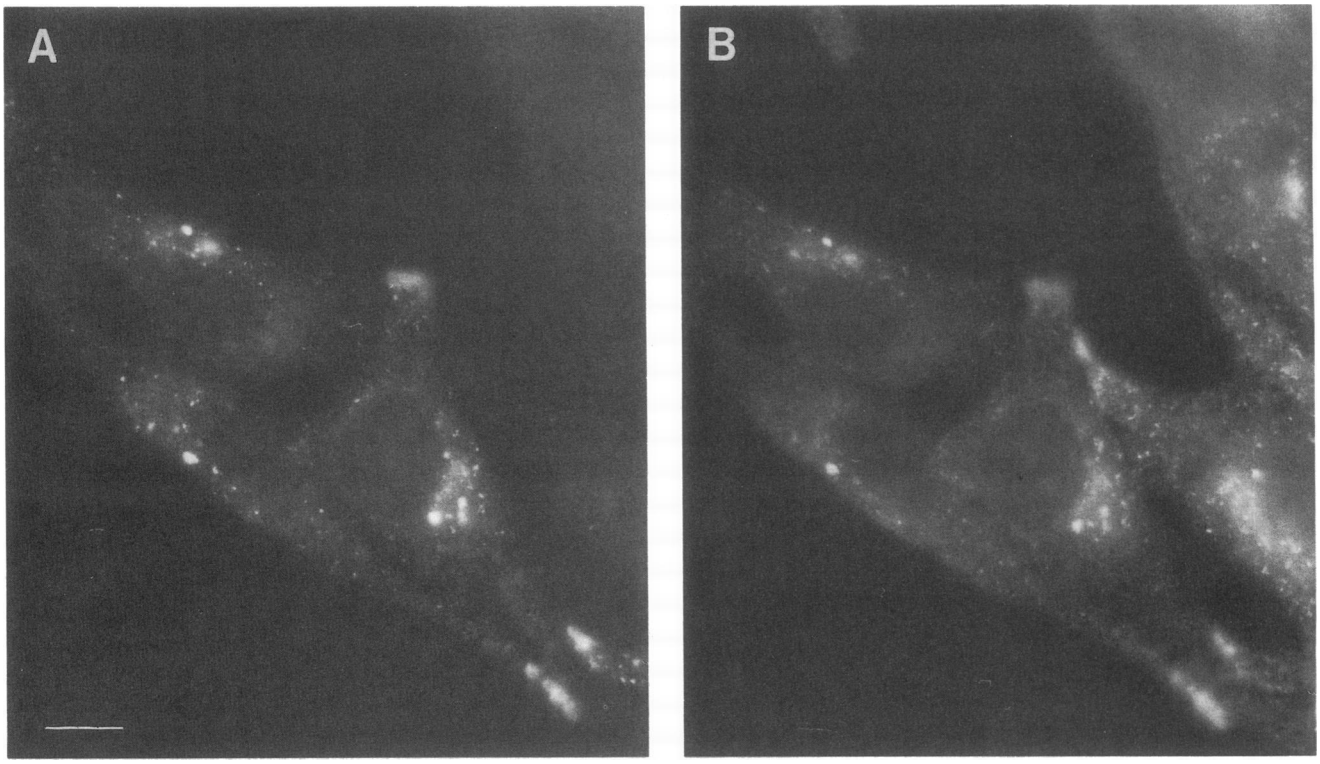

Figure 9. Immunofluorescence of HeLa cells transiently transfected with ypt5 cDNA. Cells were labeled with rabbit anti-ypt5 peptide and mouse monoclonal OKT9 anti-transferrin receptor, and then fluorescein anti-rabbit and rhodamine anti-mouse antibodies. (A) Fluorescein channel. (B) Rhodamine channel. General colocalization of the two proteins is evident. The two cells at the bottom of the field also show colocalization of ypt5 protein and transferrin receptor in enlarged endosomes at the ends of processes. Bar, 10 μm.

ypt5 locus; therefore, it would be expected that its level of expression could only be lower than that of ypt5 protein. Nevertheless, it can carry out the function of *ypt5*. Using a similar approach to that described above for rab5, the *ypt5* gene can also be replaced with another copy of itself linked to the *ura4* marker with similar efficiency (Craighead and Armstrong, unpublished data). This gene replacement system can be used to select conditional mutants of both ypt5 and rab5; it may be of interest to compare the phenotypic effects of such mutations in vivo, such as temperature sensitivity, with behavior of mutant proteins in an endosome fusion assay in vitro.

In a further test of the similarity between the two homologues, we investigated the localization of ypt5 after transient expression in mammalian cells. The protein had a vesicular distribution that generally coincided with a marker for early endosomes (Figure 9). This result suggests that ypt5 possesses similar features to rab5 for targeting to early endosomes. Chavrier *et al.* (1991) reported the effects of exchanging C-terminal portions of rab proteins on their localization and concluded that the region from residues 10 to 35 from the C-terminus of rab5 was sufficient to target fusions of rab2 and rab7 to early endosomes. Surprisingly, this is in the least-conserved region between canine rab5 and ypt5 (Figure 2). The longest region of identity is only three amino acids, one of which is not conserved in human rab5 (Zahraoui *et al.*, 1989). Perhaps the targeting "signal" is a structural motif with no exact sequence requirements; alternatively, other regions may also be involved in localization.

Thus, several independent but circumstantial lines of evidence suggest that the sequence homology between ypt5 and rab5 may reflect a similarity of function. If this is the case, the fact that ypt5 is required for a normal growth rate, and under some conditions is essential, would imply that a functional endocytic pathway is generally important for viability of eukaryotic cells. In support of this notion, Raths *et al.* (1993) have described two mutants of *S. cerevisiae* that are blocked in the early stages of endocytosis and are also temperature sensitive for growth. It is not obvious that delivery of extracellular fluid to the vacuoles would fulfill any vital metabolic role for *S. pombe* in culture, and inhibition of delivery to the vacuole of *S. cerevisiae* by disruption of the *ypt7* gene has no effect on growth (Wichmann *et al.*, 1992). Perhaps just as likely is that an endocytic pathway is required to control the balance of plasma membrane growth at different sites and stages of the cell cycle and

potentially to allow regulation of these processes independent of the rate of secretion.

To test these speculations, it will be necessary to develop an assay for endocytosis in *S. pombe*. Lucifer Yellow has been used as a marker for fluid-phase uptake into the vacuole of *S. cerevisiae* (Riezman, 1985). However, *S. pombe* takes up Lucifer Yellow (Sigma) less efficiently than *S. cerevisiae*, and under some conditions the dye can leak out of the vacuoles into the cytoplasm (Armstrong, unpublished data), raising doubts as to its reliability as a fluid-phase marker in this organism. In *S. cerevisiae*, an alternative endocytic probe is the alpha-mating factor, which binds to a receptor and passes through a vesicular intermediate en route to the vacuole (Singer and Riezman, 1990). Recently, one of the mating factors of *S. pombe* has been purified and characterized (Davey, 1992). This may provide an opportunity to assay endocytosis in *S. pombe* and to investigate by genetic approaches the role that ypt5 might play in the process.

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