CONTROL OF CELLULAR PHYSIOLOGY BY TM9 PROTEINS IN YEAST AND DICTYOSTELIUM

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ABSTRACT

TM9 proteins constitute a well-defined family, characterized by the presence of a large variable extracellular domain and of nine putative transmembrane domains. This family is highly conserved throughout evolution and comprises three members in Dictyostelium discoideum and Saccharomyces cerevisiae, and four in human and mice. In Dictyostelium, previous analysis demonstrated that TM9 proteins are implicated in cellular adhesion. In this study, we generated TM9 mutants in S. cerevisiae and analyzed their phenotype with particular attention to cellular adhesion. S. cerevisiae strains lacking any one of the three TM9 proteins were severely suppressed for adhesive growth and filamentous growth under conditions of nitrogen starvation. In these mutants, expression of the FLO11-lacZ reporter gene was strongly reduced while expression of FRE(Ty1)-lacZ was not, suggesting that TM9 proteins are implicated at a late stage of nutrient-controlled signaling pathways. We also reexamined the phenotype of Dictyostelium TM9 mutant cells, focusing on nutrient-controlled cellular functions. While the initiation of multicellular development and autophagy was unaltered in Dictyostelium TM9 mutants, nutrient-controlled secretion of lysosomal enzymes was dysregulated in these cells. These results suggest that in both yeast and amoebae, TM9 proteins participate in the control of specific cellular functions in response to changing nutrient conditions.

INTRODUCTION

TM9 proteins constitute a well-defined family of proteins characterized by the presence of nine transmembrane domains and a high degree of similarity (1). There are three members of this family in Saccharomyces cerevisiae, Dictyostelium amoebae and Drosophila flies, and four in human and mice (2). Although their high degree of evolutionary conservation suggests that they play an important role in cellular physiology, little is known about the role of TM9 proteins. The most detailed studies to date concerning the role of TM9 proteins stem from the study of Dictyostelium amoebae.

The cellular slime mold Dictyostelium discoideum has been used previously as a model organism to study phagocytosis and the endocytic pathway. During the course of a systematic search for mutants affected in phagocytosis, a mutant cell line with a defective TM9 protein (named Phg1 or Phg1a) was identified (3). Loss of Phg1a function led to a defect in cellular adhesion, resulting in inefficient phagocytosis. Although it was initially proposed that Phg1a might be an adhesion molecule (3), a more detailed analysis
suggested that it might rather indirectly affect cell adhesion by controlling the cell surface level of an as yet unidentified cell surface adhesion molecule (2). There are two other members in the TM9 family in Dictyostelium, Phg1b and Phg1c. Phg1a and Phg1b appear to play synergistic roles in the control of cell adhesion (2). In yeast or in human, the function of TM9 proteins has essentially not been studied.

To understand better the function of TM9 proteins, we analyzed the phenotypes of TM9 mutants in S. cerevisiae as well as in D. discoideum. Our results suggest that TM9 proteins play a role in late stages of a nutrient-controlled signaling cascade that ultimately controls cellular adhesion and filamentous growth in S. cerevisiae. Similarly, D. discoideum TM9 proteins, in addition to their role in cellular adhesion defect, appear to be involved in nutrient-controlled steps of intracellular transport.

**Experimental procedures**

Cells and reagents. All yeast strains used in this study were obtained in the Σ1278b genetic background and are described in Table 1. Yeast transformation was performed using the lithium acetate method (4). Each TM9 gene was deleted by PCR-mediated gene disruption, using the G418 resistance gene cassette derived from template plasmid pFA6-kanMX2 (5) (6), or the HIS3 or TRP1 gene cassette derived from template plasmids pRS303 and pRS304, respectively. Double and triple TM9 knockout mutants were obtained by crossing the single knockout strains. Yeast plasmids used in this study are described in table 2.

D. discoideum strains were grown in HL5 medium at 21°C and subcultured twice a week to maintain a maximal density of 10^6 cells/ml. All mutant strains used in this study were derived from the subclone DH1-10 (3) of the axenic Dictyostelium strain DH1, previously derived from non-axenic wild-type cells (7). For simplicity, DH1-10 cells are referred to as wild-type cells. The phg1a (3), phg1b (2), phg1a overexpressing Phg1b (2), the double phg1a/phg1b (2), phg2 (8) and apml (9) mutant strains were described previously. Rabbit polyclonal antibodies to Dictyostelium Cathepsin D (10) was a kind gift from Dr. J. Garin (CEA, Grenoble, France). The contact site A protein was detected with monoclonal antibodies 33-294-17 (11).

**Phylogenetic tree.** The phylogenetic tree of TM9 proteins in D. discoideum (Phg1a, b, c), human (TM9SF1, 2, 3 and 4) and S. cerevisiae (Tnn1, Tnn2 and Tnn3) was obtained using clustalW software from European Bioinformatics Institute (http://www.ebi.ac.uk). The corresponding accession numbers are: TM9SF1-O15321, TM9SF2(p76)-Q99805, TM9SF3 (hSMBP)-Q9HD45, TM9SF4-Q92544, Phg1a-Aj318760, Phg1b-Aj507828, Phg1c-Aj507829, Tnn1-S000004073, Tnn2-S000002514 and Tnn3-S00000915.

**Yeast adhesive and filamentous growth.** To observe adhesive growth, haploid yeast strains were plated on YPD for 4 days at 30°C. Plates were photographed before and after washing with Distilled water to visualize the remaining adherent cells. To induce filamentous growth, diploid yeast strains were grown on synthetic low-ammonia dextrose (SLAD) agar plates for 3 days at 30°C (12). Pictures of the agar plates were taken with a Zeiss Axiopt 1 equipped with an Axiocam color camera (Carl Zeiss MicroImaging Inc., Thornwood, United States). When indicated, the dominant active RAS2^val19 allele was expressed using the YEp-Ras^val19 plasmid (13). TOR1 (Target Of Rapamycin) was overexpressed using the pSEY18-TOR1 plasmid (14).

**β-Galactosidase Assays.** Expression of FLO11-lacZ and FRE(Ty1)-lacZ reporter genes was determined as previously described (15-17) by measuring β-galactosidase activity in haploid or diploid yeast strains transformed with the corresponding plasmids. FRE(Ty1)-lacZ plasmid was a kind gift from Dr. H.D. Madhani (UCSF, San Francisco, USA). Cells were grown in liquid YNB medium to exponential growth phase (8 hours), washed with breaking buffer (100 mM Tris-HCl pH 8, 20% glycerol) and pelleted. Cell pellets were resuspended with 250 µl breaking buffer containing 1 mM Dithiothreitol and 5 mM PMSF and lysed mechanically by vortexing samples with glassbeads at 4°C. Cell extracts (10 μl) were added to 200 µl Z-buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 50 mM mercaptoethanol, pH 7) and incubated for 5 min at 28°C. Enzymatic activity was revealed with 5 mM 2-Nitrophenyl-β-D-galactopyranoside (ONPG) and was stopped with 220 mM Na2CO3. The activities were normalized to the total protein in each extract using a Bio-Rad protein assay kit. β-galactosidase specific activity equals (OD_{420} x
maturation in endosomal compartments, cells were incubated in HL5 medium for three days. 10⁶ cells were harvested and centrifuged. Proteins in supernatants were precipitated with TCA. Cellular pellets and precipitated supernatants were resuspended in sample buffer (0.103 g/ml sucrose, 5x10⁻² M Tris, pH 6.8, 5x10⁻¹ M EDTA, 0.5 mg/ml bromophenol blue, 2% SDS) and proteins were separated on a 10% polyacrylamide gel and transferred onto a nitrocellulose Protran BA 85 membrane (Schleicher & Schuell, Dassel, Germany). Membranes were incubated with an anti-cathepsin D rabbit antiserum (1/1500) and then with a horseradish peroxidase-coupled donkey anti-rabbit IgG (Bio-Rad, Hercules, CA), washed, and revealed by enhanced chemiluminescence (Amersham Biosciences, UK).

To assess csA expression, 1.5x10⁶ cells were harvested and lysed in 40 µl of sample buffer. Proteins (15 µl) were separated on a 10% polyacrylamide gel in reducing conditions and transferred onto nitrocellulose. Membranes were incubated with the anti-csA antibody and then with a horseradish peroxidase-coupled donkey anti-mouse immunoglobulin (Amersham Biosciences UK), washed and revealed by enhanced chemiluminescence.

**Real Time PCR.** Cells (wild-type, phg2 cells and all the phg1 mutants) were grown in HL5 medium for three days to a final density of 10⁶ cells/ml. As a positive control, 10⁵ of each mutant cells were incubated for 6 hours in phosphate buffer to allow induction of autophagy genes. Cells were harvested and RNAs were purified with NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany). The Agilent 2100 Bioanalyzer (Agilent technologies, Santa Clara, United States) was used to assess RNA quality. cDNA was synthesized from 1 µg of total RNA using random hexamers and Superscript II reverse transcriptase (Invitrogen, Basel Switzerland). Amplicons were designed over exon boundaries using the program Primer Express v 2.0 (Applied Biosystems, Foster City, United States) with default parameters. Sequences were aligned against the Dictyostelium genome by BLAST to ensure that they were specific for the gene being tested. Oligonucleotides were obtained from Invitrogen and the sequences are the following, ATG1 (5'-aaacaaatgaaccttgtgacatt-3' and 5'-tgatctatgactgcatcactcttctc-3'), ATG8 (5'-aagcacacctgacaa-3' and 5'-gcggtaatatcagcgctgcac-3'), ATG1 (5'-aaacaaatgaaccttgtgacatt-3' and 5'-tgatctatgactgcatcactcttctc-3') and ATG9 (5'-
RESULTS

TM9 proteins in Saccharomyces cerevisiae. Three genes encoding members of the TM9 family can be identified in the budding yeast S. cerevisiae genome: YLR083c (TransMembraneNine 1 : TMN1), YDR107c (TMN2) and YER113c (TMN3). Tmn1 (also called Emp70) was previously described as an endosomal membrane protein (21) and it is 86% and 41% similar to Tmn2 and Tmn3 proteins, respectively. As described earlier (22), TM9 proteins can be separated in two groups. Group I is characterized by a conserved motif at position 50 (VGPGxNxQETY) and a short N-terminal domain (220 amino acids), while group II exhibits a characteristic sequence immediately after the signal peptide (FY(V/L)PG(VL)AP), and a longer N-terminal domain (280 amino acids). Tmn1 and Tmn2 exhibit the characteristic group II motif (FYLPGVAP and FSLPGLSP, respectively) and a long N-terminal domain (310 and 316 amino acids, respectively). Tmn3, like Dictyostelium Phg1c, does not exhibit characteristics of either group and cannot be classified unambiguously based on these criteria only.

Reconstruction of phylogenetic trees based on sequence similarities with human and Dictyostelium TM9 proteins led to the same conclusions (Fig. 1A): Tmn1 and Tmn2 are closely related to Dictyostelium Phg1a (group II); No S. cerevisiae TM9 protein could be unambiguously attributed to group I. In agreement with this, Tmn1 and Tmn2 exhibited the highest degree of identity with Dictyostelium Phg1a (Fig. 1B).

TM9 proteins are essential for yeast cell adhesion and filamentous growth. TM9 proteins play an essential role in D. discoideum cellular adhesion (2). Therefore, in budding yeast, we decided to first examine their role in adhesion. For this, we generated haploid and diploid yeast strains in the dimorphic SΣ1278b genetic background carrying deletions in the TMN1, TMN2 and TMN3 genes in all possible combinations. The single, double or triple deletion strains exhibited no obvious growth defect, suggesting that TM9 proteins are involved in a non-essential cellular function (data not shown). We also failed to detect differential growth in media containing various carbon sources including glucose, galactose, maltose, glycerol and sucrose (data not shown). Finally, no effect of TM9 mutations on cell morphology or colony shape was apparent (data not shown).

In contrast, haploid strains lacking at least one of the three TM9 proteins were severely defective for adhesive growth (Fig. 2A). Single tmn2Δ and Tmn1Δ mutant strains retained a minimal capacity to adhere to the agar surface, while no adhesion was observed in the single tmn3Δ mutant or in any of the double and triple mutants. In diploid S. cerevisiae strains, cellular adhesion is required for the development of pseudohyphal (PH) filaments in response to nitrogen starvation (12,23). Therefore, the requirement of TM9 proteins for filament formation was tested in homozygous diploid TM9 mutant strains grown on solid nitrogen starvation medium. We found that homozygous diploid single TM9 mutant strains had only a slightly reduced capacity to develop PH filaments, while the double and triple mutants were completely unable to grow in the filamentous form (Fig. 2B). Thus, although the mechanisms governing cellular adhesion in S.
cerevisiae and in D. discoideum are very different, TM9 proteins also play an essential role in cellular adhesion and filamentous growth in the budding yeast S. cerevisiae.

TM9 proteins are required for expression of FLO11 and act downstream of Ras2 and TOR. The morphogenetic switch to filamentous growth in S. cerevisiae involves the cooperation of at least two different signaling pathways, a MAP kinase cascade and a cAMP-dependent pathway (16,24-27) (Fig. 3A). A central element of these two pathways is the GTP-binding protein Ras2, which is thought to stimulate the transcription factor Ste12 via the MAP kinase pathway. In the cAMP-dependent pathway, activated Ras2 can interact with adenylate cyclase and this results in an increase of cAMP, which in turns activates cAMP-dependent protein kinases and leads to the activation of Flo8. The TOR (Target Of Rapamycin) pathway also plays a role in sensing nitrogen sources and regulating physiological responses independently of the MAP kinase and cAMP pathways (28). These pathways converge to regulate the expression of the FLO11 gene, which encodes a cell surface flocculin (Fig. 3A), which mediates cell adhesion (29,30). Numerous studies have shown specifically that in the dimorphic Σ1278b genetic background, expression of the cell surface flocculin Flo11 is essential for adhesive growth and filament formation (16,31,32). We therefore tested whether expression of FLO11 might be affected by mutations in TM9 genes. We found that in all haploid TM9 mutant strains, expression of a FLO11-lacZ reporter gene was strongly reduced when compared to a wild-type strain (Fig. 3B). Similar results were obtained with diploid strains (data not shown). Thus, TM9 proteins appear to be involved in the regulation of the expression of adhesion molecules in yeast.

To further explore the role of TM9 proteins in regulation of cellular adhesion, we performed a genetic epistasis analysis by expressing the dominant active Ras2.VΔ19 allele. As expected, this led to increased filament formation in the control strains, but this effect was completely inhibited in all TM9 double and the triple mutant strains (data not shown). Similarly, TOR1 overexpression did not restore filamentous growth in double or triple TM9 mutants (data not shown). These results indicate that TM9 proteins act downstream of the Ras/cAMP and TOR pathways to control FLO11 expression.

The activity of the MAPK pathway can be monitored by the FRE(Ty1)-lacZ reporter gene, the expression of which depends on elements of the Kss1-MAPK cascade and the combined action of the transcription factors Ste12 and Tec1 (15,17). Here, we found that expression of FRE(Ty1)-lacZ was not reduced in TM9 mutant strains (Fig. 3C), indicating that TM9 proteins do not affect FLO11 gene expression and filamentous growth by inhibiting the Kss1-MAPK pathway.

Taken together, these results suggest that in the budding yeast, TM9 proteins play a critical role in the late stages of a nutrient controlled pathway notably regulating FLO11 gene expression. These observations prompted us to investigate the possibility of a link between TM9 proteins and nutrient-controlled functions in Dictostelium.

Dictostelium Phg1 proteins are not implicated in initiation of development or of autophagy. In Dictostelium, multicellular development, autophagy and secretion of lysosomal enzymes are all critically controlled by nutrient availability. To test the potential involvement of TM9 proteins in these functions in D. discoideum, we made use of TM9 mutant cells (named phg1 or phg1a and phg1b in D. discoideum) described previously (3) and assessed nutrient-controlled functions in wild-type and mutant cells. Specifically, we examined the phenotypes of phg1a, phg1b and the double phg1a/b mutant cells. In addition, we analyzed phg1a mutant cells overexpressing Phg1b (phg1a+PHG1b). To monitor the initiation of development, we incubated wild-type or mutant cells in medium containing a defined amount of nutrients and followed the formation of multicellular aggregates. This test has proven useful to demonstrate abnormalities in the initiation of multicellular development, as observed for example in cells defective in the Phg2 kinase (20). In wild-type or in phg1 mutant cells the induction of multicellular aggregates was inhibited by a low concentration of nutrients (4 to 6% HL5 medium) (Fig. 4A). This was further confirmed by determining the expression of csA, a well-characterized marker of multicellular development (Fig. 4B).

Nutrient starvation also induces the expression of a collection of genes involved in autophagy in Dictostelium (33-36). To detect an abnormal autophagy induction by nutrients, we measured by real-time PCR the expression of three autophagy genes, ATG1, ATG8 and ATG9, in wild-type, phg2 and in various phg1 mutant cells. The expression of these marker genes was
not induced in HL5 medium in wild-type or mutant cells (Fig. 5 and supplemental Fig. S1). Thus, autophagy appears normally inhibited by nutrients in all phg1 mutant cells as well as in phg2 mutant cells. Starvation induced the expression of autophagy genes in wild-type and in phg1 mutant cells (Fig. 5 and supplemental Fig. S1), confirming that Phg1 proteins are not involved in the induction of autophagy in Dictyostelium.

Secretion of lysosomal enzymes is dysregulated in Dictyostelium TM9 mutant cells. The third phenomenon controlled by nutrients in Dictyostelium is the regulated secretion of lysosomal enzymes. Lysosomal enzymes are normally synthesized in the ER and transported to lysosomal compartments where they are activated by limited proteolysis (37). Upon starvation, cells secrete defined amounts of each lysosomal enzyme, for example a large percentage of N-acetyl β-glucosaminidase or α-mannosidase, and a more moderate fraction of the acid phosphatase pool (38). N-acetyl β-glucosaminidase, α-mannosidase and acid phosphatase activities were measured in cells and in the medium after three days of cellular growth in HL5 medium. Interestingly, in yeast, synthetic lethality has been observed between TM9 genes and YPT6 (39). Since YPT6 is involved in the function of the endocytic pathway, this suggests a link between TM9 proteins and the endocytic pathway. Wild-type cells and phg2 mutant cells secreted approximately 10% of their total lysosomal enzymes in HL5 medium. In contrast, phg1a mutant cells secreted a huge amount of hydrodases: 85% of N-acetyl β-glucosaminidase and α-mannosidase and 30% of the acid phosphatase pool were found in the extracellular medium (Fig. 6A and supplemental Table S1). This secretion was continuous and observed even immediately after cells were transferred to fresh HL5 medium (Fig. 6B), demonstrating that secretion of lysosomal enzymes in phg1a mutant cells was not inhibited by nutrients. A similar phenotype was also observed in phg1a/phg1b double knockout cells (Fig. 6). Interestingly, phg1b mutant cells also secreted abnormally lysosomal enzymes, albeit not as much as phg1a (Fig. 6). Moreover, overexpression of Phg1b in phg1a mutant cells caused a marked decrease of enzymes secretion (Fig. 6). As expected, starved wild-type and mutant cells all secreted efficiently lysosomal enzymes in the medium (supplemental Fig. S2).

Lysosomal enzymes could conceivably be secreted either directly after their passage through the Golgi apparatus, or after their targeting to lysosomal compartments. To distinguish between these two possibilities, procathepsin D and cathepsin D were detected by Western blot in cell pellets and supernatants. Phg1a mutant cells secreted cathepsin D in its mature form, while, as described previously (9), apm1 mutant cells secreted the precursor form (Fig. 6C). This defect was also complemented by overexpression of Phg1b. As expected, the double knock-out phg1a/phg1b cells secreted also mature form of cathepsin D (Fig. 6C). Together, these results demonstrate that the dysregulated secretion of lysosomal enzymes by phg1a mutant cells is not caused by a defect in targeting to the lysosomes, but rather by a defect in the regulation of lysosomal enzyme secretion. They also indicate a certain degree of redundancy between Phg1a and Phg1b for the control of lysosomal enzyme secretion in Dictyostelium, as evidenced by the partial complementation of the phenotype of phg1a mutant cells by the overexpression of Phg1b. Besides defects in lysosome enzyme secretion, we detected no general defect in the morphology of endocytic compartments labeled with antibodies against the p80 endosomal marker and the vacuolar H⁺-ATPase (40) (data not shown). Endocytosis of a fluid phase marker, as well as its subsequent recycling to the extracellular medium were also tested. Fluid phase was endocytosed in wild-type and in mutant cells at the same rate (Fig. 7A and supplemental Fig. S3). After loading cells for two hours in HL5 containing Alexa-647-coupled dextran, recycling was measured. Internalized fluorescence was released in the medium with similar kinetics in wild-type and phg1 mutant cells (Fig. 7B and supplemental Fig. S3). These results indicate that the overall organization and function of the endocytic pathway are not grossly altered in phg1a mutant cells.

**DISCUSSION**

The presence of nutrients is a major regulator of eucaryotic cell physiology. Starvation induces autophagy in many very different cellular systems ranging from mammalian cells to amoebae or yeast. In addition, it can induce more specific responses in
different systems, for example invasive growth in yeast, and in Dictyostelium regulated secretion of lysosomal enzymes and multicellular development. Our results indicate that in yeast and in Dictyostelium, a subset of these specific responses implicates TM9 proteins. Indeed, in both organisms, some nutrient-controlled responses are still normal in TM9 mutant cells (e.g. induction of the MAPK pathway by starvation in yeast, or induction of autophagy and multicellular development in starved Dictyostelium). This indicates that these cells are still able to sense the presence or absence of nutrients. However some specific responses to nutrients are affected in TM9 mutant cells, notably filamentous growth in yeast and lysosomal enzyme secretion in Dictyostelium. Our results in yeast further suggest that TM9 proteins are critical at a late stage of signal transduction, since upstream elements of the nutrient-sensing pathways (MAPK pathway) are still functional in TM9 mutants, while late stages (induction of FLO11) are defective. The most simple interpretation of these observations is that a TM9-controlled signaling pathway converges with nutrient-sensing pathways at a late stage and controls specifically a few elements of cellular physiology. According to this model, the detailed organization of the nutrient-sensing and TM9 signaling pathways in various organisms would account for the fact that synergistic or antagonistic relationships can be observed in different situations. Interestingly, in this study, we observed clear functional redundancy between TM9 proteins in yeast, as well as in Dictyostelium. This is compatible with the notion that all TM9 proteins act in at least partially overlapping signaling pathways. However, although functional redundancy can often account for wild-type phenotypes in single knockout mutants, this was not the case for single TM9 mutants, which exhibited clear phenotypes in yeast as well as in Dictyostelium. This suggests that TM9 proteins participate in a very finely tuned signaling network controlling a few critical elements of cellular physiology. More detailed studies will be necessary to determine the exact role played by TM9 proteins in cellular signal transduction pathways.

Since this study was performed in parallel in two very different organisms, it is interesting to compare the elements of the cellular physiology that are placed specifically under the control of TM9 proteins in these two situations. In yeast, filamentous growth requires the expression of specific adhesion molecules and polarized budding. It is believed to represent a coordinated program allowing invasive growth. In conditions of nutrient depletion, yeast cells can thus invade their substrate, and this can allow them to uncover new sources of nutrients. When switched to a medium containing no nutrients, Dictyostelium amoebae also undergo a series of successive changes. Within minutes, they start secreting lysosomal enzymes (38). Over a longer period of time (after a few hours), they express proteins necessary for autophagy, probably to obtain aminoacids by digesting cytosolic proteins. Finally, upon prolonged starvation (>6h) expression of appropriate genes allows the initiation of multicellular development. Remarkably, only the first part of this response (secretion of lysosomal enzymes) is affected in TM9 mutant cells: unlike wild-type cells, these mutant cells secrete mature lysosomal enzymes even in the presence of nutrients, suggesting that the dysregulated secretion of lysosomal enzymes is not caused by a defect in targeting to lysosomes, but rather by an abnormal regulation of lysosome secretion. In addition to this phenotype, previous studies have shown that adhesion of TM9 mutant cells to their substrates is modified: cells retain only the ability to adhere to certain substrates, possibly reflecting a change in their surface adhesion molecules (2,3). We would like to speculate here that the TM9-controlled response in Dictyostelium represents an invasive growth program similar to that described in yeast. Indeed, changes in cell adhesion and secretion of lysosomal enzymes should allow starved Dictyostelium cells to digest and invade their substrate, and possibly to uncover new sources of nutrients. If this hypothesis is true, we should expect future studies to reveal more common elements between the control of invasive growth in budding yeast and in Dictyostelium amoebae.
REFERENCES


**FOOTNOTES**

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**FIGURE LEGENDS**

Fig. 1. The TM9 family in *Dictyostelium discoideum* and *Saccharomyces cerevisiae.*

(A) Phylogenetic tree of TM9 proteins in *D. discoideum* (Phg1a, b, c), human (TM9SF1, 2, 3 and 4) and *S. cerevisiae* (Tmn1, Tmn2 and Tmn3). The subgroup (I, II or undetermined (?)) of each TM9 protein is indicated.

(B) Degrees of identity and similarity (bracketed) between TM9 proteins from *S. cerevisiae* and *D. discoideum.* Together these results suggest that yeast proteins Tmn1 and Tmn2 belong to the same subgroup as *D. discoideum* Phg1a.

Fig. 2. Role of TM9 proteins in cellular adhesion and filamentous growth in *S. cerevisiae.*

(A) Adhesive growth is defective in TM9 mutant yeast. Haploid yeast strains of the indicated genotype were grown on solid YPD medium for 5 days at 30°C, and plates were photographed before (total growth) and after (adhesive growth) washing non-adhesive cells off the surface.

(B) Filamentous growth of TM9 mutant cells. Diploid yeast strains of the indicated genotype were streaked on nitrogen starvation plates (SLAD) to induce filamentous growth. Pictures were taken after three days of growth at 30°C. Bar, 100 μm.

Fig. 3. Effect of TM9 mutations on gene expression.

(A) The morphogenetic switch to filamentous growth in *S. cerevisiae* involves the cooperation of different signaling pathways. A MAP kinase cascade and a cAMP-dependent pathway are controlled by the GTP-binding protein Ras2. The TOR pathway also participates in the physiological response to starvation independently of the MAP kinase or cAMP pathways. These pathways converge to regulate the expression of the *FLO11* gene, which encodes a cell surface flocculin.

(B) Role of TM9 proteins in *FLO11-lacZ* expression. Haploid yeast strains of the indicated genotype carrying plasmid B3782 (*FLO11-lacZ*) were assessed for β-galactosidase activity during growth in logarithmic phase in liquid YNB medium. The β-galactosidase activity is expressed in nanomoles per minutes per milligram of cellular proteins. The bars depict mean values +/- standard deviations of three transformants, each determined in triplicate.
Expression of FRE(Ty1)::LacZ reporter gene in TM9 mutant strains. β-galactosidase activity was measured in diploid strains carrying the plasmid pFRE(Ty1)::LacZ during growth in logarithmic phase in liquid YNB medium.

Fig. 4. Initiation of multicellular development is normal in phg1a mutant cells.
(A) Wild-type and mutant cells were placed for 24h in a medium containing a defined amount of nutrients, obtained by diluting HL5 medium with phosphate buffer. After 24h, multicellular development was monitored by assessing the presence (+) or absence (-) of tight cellular aggregates. Unlike wild-type and phg1 cells, phg2 mutant cells initiated multicellular development at high concentrations of nutrients.
(B) Cells treated as in A were harvested and the expression of Contact site A (csA) was determined by Western blot analysis.

Fig. 5. Phg1 mutant cells induce normal transcription of the ATG8 autophagy gene.
Wild type and mutant cells were grown in HL5 medium for three days to a final density of 10⁶ cells/ml. Cells were then harvested and RNA samples extracted. Expression of ATG8 was quantified by real-time PCR. Alternatively, cells were starved in phosphate buffer (SB) for 6 hours to induce expression of ATG8. Similar results were obtained when the expression of ATG1 and ATG9 was assessed (Fig. S1).

Fig. 6. Phg1 mutant cells secrete mature lysosomal enzymes in rich medium.
(A) Wild type or mutant cells were grown in HL5 medium for three days to a final density of 2x10⁶ cells/ml. Cells were harvested and centrifuged, and the activity of lysosomal enzymes determined in cell pellets and in supernatants. The fraction of enzymatic activity found in the supernatant is indicated. The total amount of enzymatic activity (secreted + intracellular) was similar in all strains analyzed (Table S1). NAG: N-acetyl β-glucosaminidase; MAN: α-mannosidase; AP: acid phosphatase. The results presented are the average and SEM of three independent experiments. *: p<0.01 (student t-test).
(B) Wild-type or mutant cells were collected and incubated in fresh HL5 medium. After 0, 1, 2, 3 h, the NAG activity was determined and expressed as described above.
(C) Cells were grown and processed as described in A. Procathepsin D (im., 53kDa) and cathepsin D (mat., 44 kDa) were detected by Western blot in the cell pellet (P) and in the medium (SN). In wild-type and phg2 cells, mature cathepsin was retained in the cells. In phg1a and in phg1a/phg1b mutant cells, mature cathepsin D was secreted in the extracellular medium. For comparison, apm1 mutant cells in which targeting to the lysosomes is defective, secreted immature procathepsin D.

Fig. 7. Fluid phase uptake and recycling are not altered in phg1 mutant cells.
(A) Wild-type and mutant cells were incubated for 1 hour in HL5 medium containing 10 µg/ml of fluorescent dextran. Internalized fluorescence was measured by flow cytometry. (B) Cells were incubated in HL5 containing fluorescent dextran for 2 hours, then washed and transferred to fresh HL5 medium for the indicated time. Internal fluorescence was measured at each time point and expressed as a percentage of the signal at time 0. Recycling of fluid phase to the extracellular medium was similar in wild-type and mutant cells. Similar results were obtained for phg1a cells overexpressing Phg1b, and for phg2 mutant cells (Fig. S3).
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<td>MATa ura3-52 leu2 trp1</td>
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<td>YRF1</td>
<td>MATa ura3-52 leu2 his3 Tmn1Δ::kanMX</td>
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</tr>
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<td>YRF10</td>
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<tr>
<td>YRF3</td>
<td>MATa ura3-52 leu2 his3 tmn3Δ::HIS3</td>
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</tr>
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<td>YRF 100</td>
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</tr>
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<td>YRF11</td>
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<td>YRF33</td>
<td>MATa/MATa ura3-52/ura3-52 leu2/leu2 his3/his3 tmn3Δ::HIS3/tmn3Δ::HIS3</td>
<td>&quot;</td>
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<td>YRF44</td>
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<td>&quot;</td>
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<tr>
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<td>MATa/MATa ura3-52/ura3-52 leu2/leu2 Tmn1Δ::kanMX/Tmn1Δ::kanMX tmn3Δ::HIS3/tmn3Δ::HIS3</td>
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<tr>
<td>YRF77</td>
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<td>&quot;</td>
</tr>
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</table>

Table 1. Yeast strains used in this study.
Table 2. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFA6-kanMX2</td>
<td>kanr fused to TEF promoter and terminator in pFA6</td>
<td>(5)</td>
</tr>
<tr>
<td>pRS303</td>
<td>pBluescript, <em>HIS3</em></td>
<td>(41)</td>
</tr>
<tr>
<td>pRS304</td>
<td>pBluescript, <em>TRP1</em></td>
<td>(41)</td>
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<tr>
<td>pYEp213-Ras2-Val^{19}</td>
<td>pYEp213::*RAS2Val^{19} (2\mu, LEU2, amp')</td>
<td>(13)</td>
</tr>
<tr>
<td>pSEY18-TOR1</td>
<td>pSEY18::*TOR1-1 (2\mu, URA3, amp')</td>
<td>(14)</td>
</tr>
<tr>
<td>pFRE(Ty1)::LacZ</td>
<td>pLG669-Z::FRE(Ty1) (2\mu, URA3, amp')</td>
<td>(17)</td>
</tr>
<tr>
<td>B3782</td>
<td>3kb FLO11-promoter fragment in YEp355</td>
<td>(16)</td>
</tr>
</tbody>
</table>

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Figure 1

A

Subgroup

Sc Tmn1  II
Sc Tmn2  II
Dd Phg1a  II
Hs TM9SF2  II
Hs TM9SF4  II
Sc Tmn3  ?
Dd Phg1c  ?
Dd Phg1b  |
Hs TM9SF1  |
Hs TM9SF3  |

B

<table>
<thead>
<tr>
<th></th>
<th>Dd Phg1a</th>
<th>Dd Phg1b</th>
<th>Dd Phg1c</th>
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</thead>
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<tr>
<td>Sc Tmn1</td>
<td>35 (52)</td>
<td>25 (44)</td>
<td>25 (43)</td>
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<tr>
<td>Sc Tmn2</td>
<td>33 (51)</td>
<td>24 (43)</td>
<td>29 (47)</td>
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<tr>
<td>Sc Tmn3</td>
<td>28 (46)</td>
<td>22 (40)</td>
<td>20 (38)</td>
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</tbody>
</table>
Figure 3

A

Nitrogen deprivation

Ras2

MAPK (FRE(Ty1))

cAMP

TOR

Filamentous growth (Flo11)

B

FLO11-lacZ

\[ \text{β-gal activity (nmol/min/mg)} \]

C

FRE(Ty1)-lacZ

\[ \text{β-gal activity (nmol/min/mg)} \]
Figure 4

A

<table>
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<th>HL5 (%)</th>
<th>100</th>
<th>25</th>
<th>20</th>
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<th>11</th>
<th>6</th>
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<tr>
<td>phg1a+B</td>
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<tr>
<td>phg1a/b</td>
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</tbody>
</table>

B

<table>
<thead>
<tr>
<th>HL5 (%)</th>
<th>100</th>
<th>20</th>
<th>11</th>
<th>2</th>
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<tbody>
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<td>WT</td>
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<tr>
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<tr>
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<td>phg1a/b</td>
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<tr>
<td>phg2</td>
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</tr>
</tbody>
</table>
Figure 5

ATG8 expression (A.U.)

- HL5
- SB

WT phg1a phg1b phg1a+b phg1ab phg2
Figure 6

A

Secreted enzymes (% of total)

* * *

NAG
MAN
AP

WT phg1b phg1a phg1a+B phg1a+b phg2

B

NAG secretion (% of total)

WT phg1a+B
phg1a phg1a/b
phg1b phg2

0 25 50 75

Time (hours) 0 1 2 3

C

SN

im. mat.

P

im. mat.
Control of cellular physiology by TM9 proteins in yeast and dictyostelium
Romain Froquet, Nathalie Cherix, Raphael Birke, Mohammed Benghezal, Elisabetta Cameroni, François Letourneur, Hans-Ulrich Mosch, Claudio De Virgilio and Pierre Cosson

J. Biol. Chem. published online January 3, 2008

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