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The TOR nutrient signalling pathway phosphorylates NPR1 and inhibits turnover of the tryptophan permease

Anja Schmidt, Thomas Beck, Antonius Koller[1], Jeannette Kunz[2] and Michael N.Hall[3]

Department of Biochemistry, Biozentrum, University of Basel, CH-4056 Basel, Switzerland

[1]Present address: Department of Biology, University of California, San Diego, La Jolla, CA 92093-0322, USA
[2]Present address: Department of Pharmacology, University of Wisconsin, Madison, WI 53706, USA

[3]Corresponding author
e-mail: hall@ubaclu.unibas.ch

A.Schmidt and T.Beck contributed equally to this work

The *Saccharomyces cerevisiae* targets of rapamycin, TOR1 and TOR2, signal activation of cell growth in response to nutrient availability. Loss of TOR or rapamycin treatment causes yeast cells to arrest growth in early G_1 and to express several other physiological properties of starved (G_0) cells. As part of this starvation response, high affinity amino acid permeases such as the tryptophan permease TAT2 are targeted to the vacuole and degraded. Here we show that the TOR signalling pathway phosphorylates the Ser/Thr kinase NPR1 and thereby inhibits the starvation-induced turnover of TAT2. Overexpression of NPR1 inhibits growth and induces the degradation of TAT2, whereas loss of NPR1 confers resistance to rapamycin and to FK506, an inhibitor of amino acid import. NPR1 is controlled by TOR and the type 2A phosphatase-associated protein TAP42. First, overexpression of NPR1 is toxic only when TOR function is reduced. Secondly, NPR1 is rapidly dephosphorylated in the absence of TOR. Thirdly, NPR1 dephosphorylation does not occur in a rapamycin-resistant *tap42* mutant. Thus, the TOR nutrient signalling pathway also controls growth by inhibiting a stationary phase (G_0) programme. The control of NPR1 by TOR is analogous to the control of p70 s6 kinase and 4E-BP1 by mTOR in mammalian cells.

Keywords: $p70^{s6k}$/rapamycin/signal transduction/starvation/TAP42

Introduction

The *Saccharomyces cerevisiae* TOR proteins, TOR1 and TOR2, are phosphatidylinositol kinase homologues first identified as the targets of the immunophilin–immunosuppressant complex FKBP–rapamycin (Heitman *et al*., 1991a; Kunz *et al*., 1993; Cafferkey *et al*., 1994; Helliwell *et al*., 1994). Mutations in either *TOR1* or *TOR2* (*TOR1-1* and *TOR2-1*) confer rapamycin resistance by eliminating the binding of the FKBP–rapamycin complex to TOR1 and TOR2, respectively (Heitman *et al*., 1991a; Stan *et al*., 1994; Zheng *et al*., 1995). TOR2 has two essential signalling functions. One function, which is unique to TOR2, is to control the cell cycle-dependent organization of the actin cytoskeleton via a Rho-type GTPase switch (Schmidt *et al*., 1996, 1997; Bickle *et al*., 1998). The second TOR2 function, which is shared with TOR1, is to signal activation of translation initiation and early G_1 progression in response to nutrients (Kunz *et al*., 1993; Helliwell *et al*., 1994, 1998; Barbet *et al*., 1996). TOR controls translation initiation by stimulating the association of type 2A and type 2A-related phosphatases with TAP42 (Di Como and Arndt, 1996). The mechanism by which TOR mediates the association of the phosphatases with TAP42, or how TAP42 controls translation initiation, is not known. This signalling pathway, like TOR itself, appears to be conserved in mammalian cells (Beretta *et al*., 1996; von Manteuffel *et al*., 1996; Murata *et al*., 1997; Thomas and Hall, 1997; Chen *et al*., 1998; Hara *et al*., 1998).

Combined deletion of *TOR1* and *TOR2*, or rapamycin treatment, causes yeast cells to arrest growth in early G_1 (G_0), undergo a severe reduction in protein synthesis, accumulate the storage carbohydrate glycogen, acquire thermotolerance, enlarge the vacuole, transcriptionally induce specific genes while repressing others and induce autophagy (Kunz *et al*., 1993; Helliwell *et al*., 1994; Barbet *et al*., 1996; Noda and Ohsumi, 1998). These phenotypes collectively define stationary phase, or G_0, which yeast cells enter upon starvation. Thus, rapamycin induces a starvation response. As part of the starvation response, rapamycin treatment also causes a severe decrease in amino acid import by inducing the ubiquitin-dependent degradation of high affinity amino acid permeases, including the tryptophan transporter TAT2 (T.Beck, A.Schmidt and M.N.Hall, submitted). It is not known how TOR prevents the degradation of these amino acid permeases in the presence of nutrients.

Cell surface permeases mediate the uptake of nutrients and are thus essential for cell growth and viability. In yeast, several amino acid permeases, including the general amino acid permease GAP1 and the proline permease PUT4, are tightly regulated with respect to the quality of the external nitrogen source (Sophianopoulou and Diallinas, 1995). The putative Ser/Thr kinase NPR1 (nitrogen permease reactivator) has been implicated in the activation of GAP1 which occurs when cells are shifted from a good to a poor nitrogen source (e.g. from ammonium to proline or urea) (Grenson, 1983a,b; Vandenbol *et al*., 1987, 1990). In the presence of a poor nitrogen source, NPR1 promotes GAP1 function presumably by counteracting the nitrogen permease inhibitor, NPI1 (Grenson, 1983a,b). NPI1 is an essential ubiquitin–protein ligase that mediates the ubiquitination and degradation of

GAP1 when cells are shifted to a good nitrogen source (Hein *et al*., 1995; Springael and Andre, 1998). Since dephosphorylation of GAP1 precedes degradation of the permease, it is thought that NPR1 phosphorylates and thereby protects GAP1 from degradation (Stanbrough and Magasanik, 1995). It is not known why NPR1 cannot protect GAP1 in the presence of a good nitrogen source, but it has been speculated that the kinase is not active, possibly degraded, under these conditions (Vandenbol *et al*., 1990). So far, it appears that NPR1 acts exclusively on the nitrogen source-regulated amino acid permeases and has no role in the regulation of other amino acid permeases. We have found recently that permeases previously referred to as the constitutive amino acid permeases (permeases other than GAP1 and PUT4) are also regulated. The tryptophan permease TAT2 is ubiquitinated, targeted to the vacuole and degraded upon nitrogen or carbon starvation, or when cells are shifted to a poor nitrogen source (T.Beck, A.Schmidt and M.N.Hall, submitted). These findings indicate that GAP1 and TAT2 are regulated inversely, possibly to optimize import with regard to the quality and quantity of nutrients in the medium (T.Beck, A.Schmidt and M.N.Hall, submitted). However, it is not known how the inverse regulation is achieved, and what the signalling pathway is that controls TAT2 stability or degradation.

Here we describe a signalling pathway that prevents TAT2 degradation in the presence of nutrients. Whereas NPR1 is a positive regulator of GAP1, we find that it is a negative regulator of TAT2. Furthermore, TOR and TAP42 control the phosphorylation and activity of NPR1. These findings provide a mechanism by which NPR1 is regulated, and account for the inverse regulation of GAP1 and TAT2.

Results

NPR1 and TOR have antagonistic functions

We have demonstrated recently that the tryptophan permease TAT2 is degraded when cells are starved for nutrients, i.e. when TOR is inactivated (T.Beck, A.Schmidt and M.N.Hall, submitted). Our data have suggested that TAT2 and GAP1 are regulated inversely; however, it is not known how this inverse regulation is achieved and how it is controlled by the TOR signalling pathway. We focused on NPR1 because activation of GAP1 involves NPR1 (Grenson, 1983a,b; Vandenbol *et al*., 1987, 1990), and because we isolated *NPR1* as a dosage suppressor of *TOR2-1* rapamycin resistance during our early attempts to isolate the *TOR2* gene (Kunz *et al*., 1993). We asked whether NPR1 is involved in the down-regulation of TAT2 when TOR is turned off. According to such a model, NPR1 and TOR would have antagonistic functions.

To determine whether NPR1 and TOR have antagonistic functions, we investigated genetic interactions between *NPR1* and *TOR*. First, we examined whether overexpression of *NPR1* causes a growth defect in strains with reduced TOR function. A multicopy plasmid containing *NPR1* (pNPR1) and an empty vector was introduced into a rapamycin-resistant *TOR2-1* strain (JH12-17b). The mutant *TOR2-1* allele encodes a protein that is insensitive to rapamycin; however, in a *TOR2-1* strain, TOR1 is still a target for the drug. Transformants were streaked on

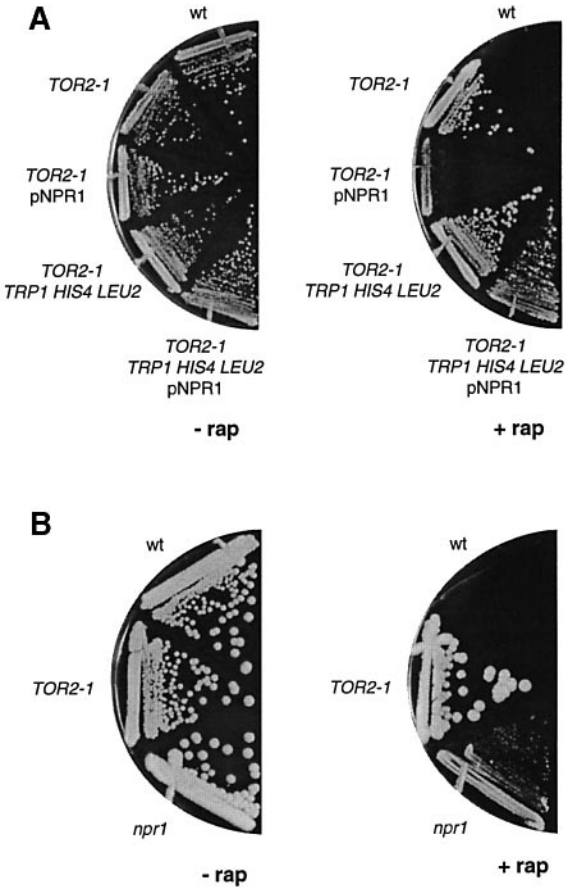

Fig. 1. NPR1 and TOR have antagonistic functions. (**A**) Overexpression of *NPR1* suppresses the rapamycin resistance phenotype of an auxotrophic *TOR2-1* strain. Auxotrophic (*TOR2-1*) (JH12-17b) and prototrophic (*TOR2-1 TRP1 HIS4 LEU2*) (AS42-4b) *TOR2-1* strains transformed with either pNPR1 or an empty vector, and our wild-type strain (wt) (JK9-3d**a**) were streaked on YPD (–rap) and on YPD containing 200 ng/ml rapamycin (+rap), and incubated for 3 days at 30°C. (**B**) Disruption of *NPR1* confers resistance to rapamycin. Wild-type (wt) (JK9-3d**a**), *TOR2-1* (JH12-17b) and *npr1* (TK54-1b) strains were streaked on YPD (–rap) and on YPD containing 200 ng/ml rapamycin (+rap), and incubated for 4 days at 30°C.

medium containing rapamycin (200 ng/ml) to inhibit wild-type TOR1. As seen in Figure 1A, overexpression of *NPR1* was toxic when TOR1 was inactivated (in the presence of rapamycin). In the absence of rapamycin, overexpression of *NPR1* did not inhibit the growth of the *TOR2-1* strain. Similarly, overexpression of *NPR1* also suppressed the rapamycin resistance of a *TOR1-1* strain (JH11-1c), but not of a *TOR1-1 TOR2-1* (JH18-3b) strain in which both TORs are rapamycin insensitive (data not shown). The above results indicate that NPR1 is able to suppress growth when one of the TORs is inhibited. Therefore, overexpression of *NPR1* should also inhibit the growth of a *TOR1* deletion strain. To test this, pNPR1 and an empty vector were transformed into strains lacking TOR1 (*tor1 TOR2* and *tor1 TOR2-1* strains MH349-3b and AS63-1d, respectively). Overexpression of *NPR1* caused a severe growth defect in strains lacking TOR1 (data not shown). Thus, overexpression of *NPR1* inhibits growth of yeast cells that have reduced TOR function.

Next, we investigated whether loss of *NPR1* restored growth in cells lacking TOR function. *NPR1* was disrupted

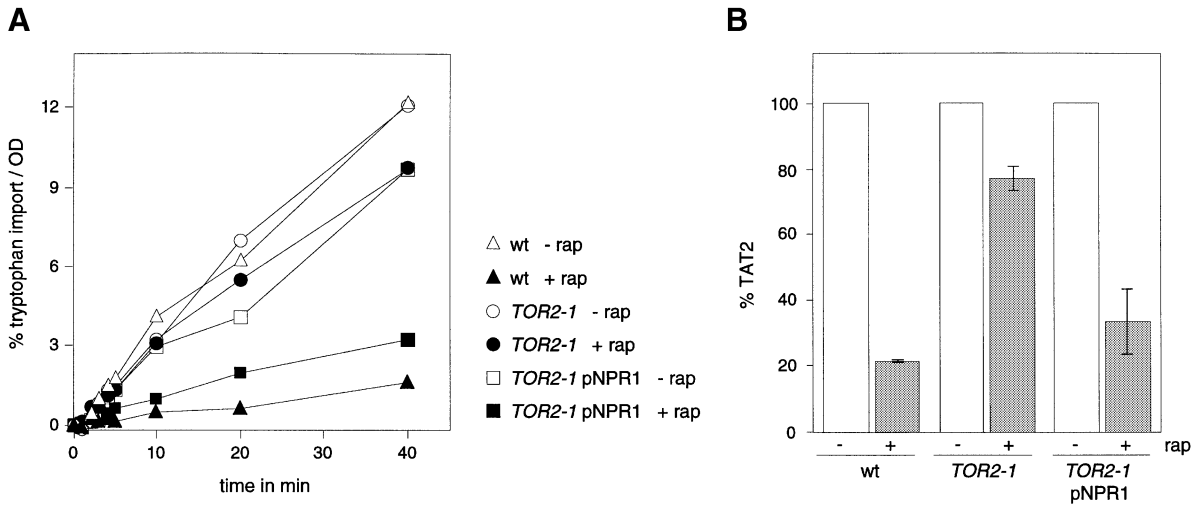

Fig. 2. NPR1 is a negative regulator of the tryptophan permease TAT2. (**A**) Overexpression of *NPR1* inhibits tryptophan import in a rapamycin-treated *TOR2-1* strain. Import rates of radiolabelled tryptophan into wild-type (wt) (JK9-3d**a**) and *TOR2-1* (JH12-17b) strains transformed with pNPR1 or an empty vector, in the absence (open symbols) and presence (solid symbols) of rapamycin (200 ng/ml). (**B**) TAT2 protein levels are decreased upon overexpression of *NPR1* in rapamycin-treated *TOR2-1* cells. Protein extracts from wild-type cells expressing HA-TAT2 (wt) (JK9-3d**a**/pHA-TAT2), and from *TOR2-1* cells expressing HA-TAT2 and carrying either pNPR1 (*TOR2-1*/pNPR1) or an empty vector (*TOR2-1*), grown in the presence or absence of rapamycin (rap), were prepared and subjected to Western analysis using anti-HA antibody. The amount of HA-TAT2 in rapamycin-treated cells is expressed as a percentage of the amount of HA-TAT2 found in rapamycin-untreated cells. Values are the average of three independent experiments.

(see Materials and methods) and the resulting *npr1* strain (TK54-1b) was streaked on medium containing rapamycin. After 3 days of incubation at 30°C, the *npr1* strain but not the wild-type parent strain formed colonies (Figure 1B). However, the resistance of the *npr1* cells to rapamycin was weaker than the drug resistance of a *TOR2-1* or *TOR1-1* strain.

We also tested whether loss of *NPR1* suppressed the slight growth defect of a *tor1* strain at 24°C (Helliwell *et al*., 1994). Haploid segregants obtained from a cross (AS60) between *TOR1 npr1* strain TK54-1b and *tor1 NPR1* strain MH349-3b were incubated on YPD at 24°C. While *tor1 NPR1* segregants exhibited a slight growth defect, as observed previously, *tor1 npr1* cells grew normally, like *TOR1 NPR1* or *TOR1 npr1* cells (data not shown). The above findings that loss of NPR1 suppresses a TOR deficiency whereas overexpression of NPR1 exacerbates a TOR deficiency suggest that NPR1 and TOR have antagonistic functions.

NPR1 is a negative regulator of the tryptophan permease TAT2

To investigate whether NPR1 and TOR have antagonistic functions in the control of TAT2, we measured tryptophan uptake in wild-type and *TOR2-1* cells transformed with pNPR1 or an empty vector. Transformants were grown at 30°C and then treated with rapamycin for 3 h. As shown in Figure 2A, tryptophan import was severely reduced in rapamycin-treated *TOR2-1* cells overexpressing *NPR1*, compared with rapamycin-treated *TOR2-1* cells that contained the empty plasmid. Overexpression of *NPR1* did not significantly reduce tryptophan uptake of *TOR2-1* cells in the absence of rapamycin. This suggests that NPR1 negatively regulates tryptophan import when TOR function is reduced.

To investigate whether loss of *NPR1* leads to an increase in amino acid import, we assessed the growth of an *npr1* strain on medium containing the immunosuppressive drug FK506 (see Materials and methods). FK506 inhibits the uptake of tryptophan (Heitman *et al*., 1993) and, consequently, auxotrophic yeast cells (*trp1*) are FK506 sensitive whereas tryptophan prototrophs (*TRP1*) or cells overexpressing the tryptophan permease TAT2 are FK506 resistant (Heitman *et al*., 1993; Schmidt *et al*., 1994; Koller *et al*., 1996). An *npr1 trp1* strain (TK109) was FK506 resistant whereas the parental *NPR1 trp1* strain (JK9-3d**a**/α) was FK506 sensitive (data not shown). This suggests that tryptophan import is increased in the absence of NPR1.

The above findings raised the question of whether the inhibition of tryptophan import and the growth defect caused by overexpression of *NPR1* are related. To test this, we asked whether overexpression of *NPR1* also suppresses the rapamycin resistance of an amino acid prototrophic *TOR2-1* strain. pNPR1 or an empty vector were introduced into the prototrophic *TOR2-1* strain (AS42-2b), and transformants were incubated on plates containing rapamycin. The prototrophic *TOR2-1*/pNPR1 cells grew better than the auxotrophic *TOR2-1*/pNPR1 cells (Figure 1A). This suggests that overexpression of *NPR1* inhibits the growth of cells, with reduced TOR function, by inhibiting tryptophan import.

We next examined whether the inhibition of tryptophan import upon overexpression of *NPR1* was due to a decrease in the amount of TAT2 protein. A plasmid expressing functional, N-terminally hemagglutinin (HA)-tagged TAT2 (pHA-TAT2) was introduced into wild-type, *TOR2-1* and *TOR2-1*/pNPR1 cells. Transformants were grown to early logarithmic phase at 30°C and then treated with rapamycin for 2 h. Cells were harvested, lysed, and protein extracts were subjected to SDS–PAGE and Western analysis. As shown in Figure 2B, extracts from rapamycin-treated *TOR2-1*/pNPR1 cells showed a decrease in the amount of TAT2 protein compared with extracts from rapamycin-treated *TOR2-1* cells transformed with an empty vector.

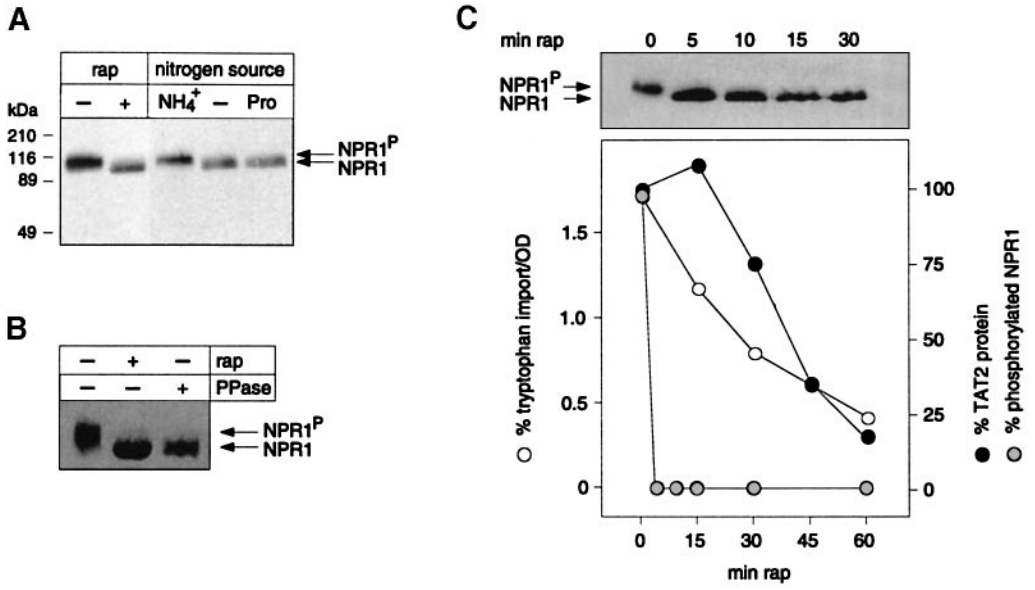

Fig. 3. TOR controls the phosphorylation of NPR1. (**A**) NPR1 from rapamycin-treated or nitrogen-starved cells migrates faster. Wild-type cells expressing HA-NPR1 (JK9-3d**a**/pHA-NPR1) were treated (+) or not treated (–) with rapamycin (rap), or grown in minimal medium containing ammonium as a nitrogen source and shifted to medium containing either ammonium (NH_4^+), no additive (–) or proline (Pro) as a nitrogen source, for 1 h. Protein extracts were prepared and subjected to Western analysis using anti-HA antibody. (**B**) NPR1 from non-starved cells is phosphorylated. Protein extracts were prepared from wild-type cells expressing HA-NPR1 (JK9-3d**a**/pHA-NPR1) and treated (+) or not treated (–) with rapamycin (rap). Protein extracts from rapamycin-untreated cells were treated (+) or not treated (–) with alkaline phosphatase (PPase). (**C**) Dephosphorylation of NPR1 upon rapamycin treatment precedes inactivation of TAT2. Wild-type cells expressing HA-NPR1 (JK9-3d**a**/pHA-NPR1) were grown to early logarithmic phase, and aliquots were removed at the indicated time points following rapamycin addition and assayed for the phosphorylation state of HA-NPR1 (NPR1) by Western analysis. The graph is a quantitation of NPR1 (shaded circles) shown in the Western blot, combined with time course data from T.Beck, A.Schmidt and M.N.Hall (submitted) on TAT2 protein levels (solid circles) and tryptophan uptake (open circles) in rapamycin-treated cells.

Thus, overexpression of *NPR1* causes a reduction in the level of TAT2 permease, presumably by inducing the degradation of TAT2.

TOR controls the phosphorylation of NPR1

NPR1 activates GAP1 only under poor nutrient conditions (Grenson, 1983a,b). It is not known how NPR1 activity is regulated but, since the N-terminus contains several PEST regions, it has been speculated that NPR1 is regulated at the level of protein stability and is degraded under good nutrient conditions (Vandenbol *et al*., 1990). In addition, our finding that the inhibitory effect of *NPR1* overexpression on TAT2 protein levels is observed only when TOR function is reduced suggests that NPR1 might be regulated by TOR.

To investigate the regulation of NPR1, we constructed a plasmid expressing functional N-terminally HA-tagged NPR1 (pHA-NPR1) and introduced it into our wild-type strain (see Materials and methods). Cells were grown in the presence of nutrients (with ammonium as a nitrogen source) and then starved for 1 h, either by addition of rapamycin or by shifting the cells to synthetic medium without ammonium or with proline as a nitrogen source. Whole cell extracts were prepared and subjected to SDS–PAGE and Western analysis. As can be seen in Figure 3A, the amount of the NPR1 protein did not increase under starvation conditions, suggesting that NPR1 is not regulated at the level of protein stability. However, the NPR1 protein from non-starved cells migrated more slowly than NPR1 from starved or rapamycin-treated cells, suggesting that NPR1 might be regulated by a TOR-dependent post-translational modification.

mTOR in mammalian cells controls its downstream effectors $p70^{s6k}$ and 4E-BP1 via phosphorylation (Thomas and Hall, 1997). To investigate whether the TOR-dependent slower electrophoretic mobility of NPR1 was due to phosphorylation, whole-cell extracts from non-starved cells were treated with alkaline phosphatase (see Materials and methods). Phosphatase-treated NPR1 from non-starved cells migrated like the faster migrating NPR1 from starved or rapamycin-treated cells (Figure 3B). Thus, TOR causes the phosphorylation of NPR1 in response to nutrients.

If there is a causal relationship between NPR1 dephosphorylation and TAT2 down-regulation, NPR1 dephosphorylation should precede TAT2 down-regulation. An inhibition of tryptophan import is observed 15 min after the addition of rapamycin (see Figure 3C) (T.Beck, A.Schmidt and M.N.Hall, submitted). We examined the kinetics of NPR1 dephosphorylation following the addition of rapamycin. As shown in Figure 3C, NPR1 was completely dephosphorylated 5 min after the addition of rapamycin. Thus, the dephosphorylation of NPR1 precedes the inactivation of TAT2, suggesting that there is a causal relationship between NPR1 dephosphorylation and TAT2 down-regulation and that TOR is preventing TAT2 down-regulation by maintaining NPR1 in a phosphorylated state. Our previous finding that TOR and NPR1 have antagonistic functions suggests that TOR is inactivating NPR1 via this phosphorylation.

TAP42 controls TAT2 stability and NPR1 phosphorylation

Di Como and Arndt (1996) have shown that TOR controls translation initiation, in response to nutrients, via TAP42.

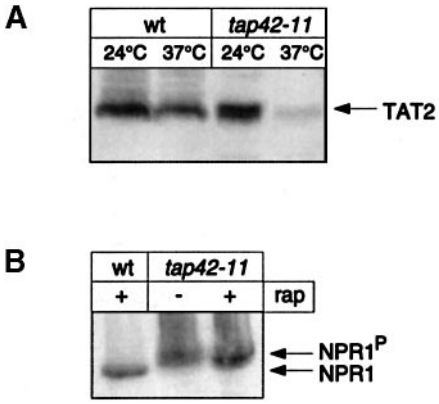

Fig. 4. TAP42 controls TAT2 stability and NPR1 phosphorylation. (**A**) TAT2 stability is controlled by TAP42. Wild-type (wt) (JK9-3d**a**) and *tap42-11* (TS19-4c) cells transformed with pHA-TAT2 were grown to early logarithmic phase at 24°C and shifted to 37°C for 2 h. Protein extracts were prepared and the levels of TAT2 protein were monitored by SDS–PAGE and Western analysis. (**B**) NPR1 phosphorylation is controlled by TAP42. Wild-type (JK9-3d**a**) and *tap42-11* (TS19-4c) cells transformed with pHA-NPR1 were grown to early logarithmic phase at 24°C and treated (+) or not treated (–) with rapamycin (rap) for 15 min. Protein extracts were prepared and the phosphorylation state of NPR1 was monitored by SDS–PAGE and Western analysis.

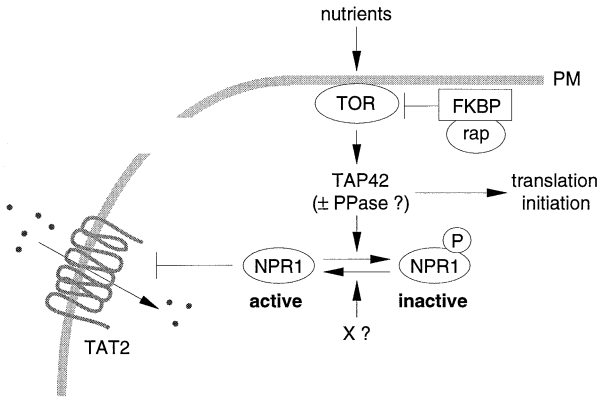

Fig. 5. Model of the TOR signalling pathway preventing degradation of TAT2 by keeping NPR1 in a phosphorylated state. TOR and TAP42 also control translation initiation. The phosphatase (X) that mediates the dephosphorylation and activation of NPR1 in the absence of nutrients is unknown. TOR is TOR1 or TOR2. rap, rapamycin; FKBP, FK506-binding protein; PPase, type 2A and type 2A-related phosphatases; PM, plasma membrane.

To investigate whether TOR requires TAP42 also to prevent the degradation of TAT2, we examined the level of TAT2 protein in a temperature-sensitive *tap42-11* mutant shifted to non-permissive temperature. At non-permissive temperature, the *tap42-11* mutation confers a recessive phenotype similar to that caused by a TOR deficiency or rapamycin treatment. At permissive temperature, *tap42-11* confers dominant rapamycin resistance (Di Como and Arndt, 1996). Wild-type and *tap42-11* mutant cells (TS19-4c) transformed with pHA-TAT2 were grown to early logarithmic phase at permissive temperature and shifted to non-permissive temperature for 2 h. Extracts were prepared and subjected to Western analysis. As shown in Figure 4A, TAT2 protein levels decreased in the *tap42-11* mutant, but not in the wild-type strain, upon shift to the non-permissive temperature. Thus, both TOR and TAP42 are required to prevent the degradation of TAT2.

The above results suggest that TAP42 might control the phosphorylation of NPR1. To investigate this, we examined if rapamycin induced the dephosphorylation of NPR1 in the *tap42-11* mutant at permissive temperature. The *tap42-11* strain (TS19-4c) was transformed with the plasmid encoding HA-tagged NPR1 (pHA-NPR1) and treated with rapamycin for 20 min. Whole-cell extracts were prepared and the phosphorylation state of NPR1 was analysed by Western blotting. As shown in Figure 4B, rapamycin failed to induce the dephosphorylation of NPR1 in the *tap42-11* strain. This suggests that TAP42, like TOR, also prevents the degradation of TAT2 by maintaining NPR1 in a phosphorylated state.

Discussion

We have shown that TOR (TOR1 and TOR2) and TAP42 control the phosphorylation of the Ser/Thr kinase NPR1 and the stability of the tryptophan permease TAT2. Under conditions of TOR inactivation (starvation or rapamycin treatment), NPR1 rapidly becomes dephosphorylated and TAT2 subsequently is degraded. Our results also suggest that dephosphorylated NPR1 mediates the degradation of TAT2. Di Como and Arndt (1996) have shown that TAP42 is a downstream effector of TOR. The simplest model to account for these findings is shown in Figure 5. According to this model, in the presence of nutrients, TOR activates TAP42 which in turn leads to the phosphorylation and inactivation of NPR1. In the absence of nutrients, TOR and TAP42 are inactive, which results in the rapid dephosphorylation and activation of NPR1 which, in turn, leads to the degradation of TAT2. The turnover of TAT2 is part of the stationary phase (G_0) programme that yeast cells normally enter upon starvation. Thus, by phosphorylating and inactivating NPR1, the TOR nutrient signalling pathway is controlling cell growth by inhibiting a stationary phase programme in addition to activating, as shown previously, a growth programme. The growth programme that is activated by TOR includes protein synthesis and the cell-cycle-dependent organization of the actin cytoskeleton (Barbet *et al.*, 1996; Schmidt *et al.*, 1996, 1997; Helliwell *et al.*, 1998). Interestingly, by preventing the turnover of amino acid permeases such as TAT2, TOR is ensuring the availability of amino acids and thereby further contributing to protein synthesis.

How is TAP42 controlling the phosphorylation of NPR1? TOR signals to the translation initiation machinery by stimulating the association of type 2A and type 2A-related phosphatases with TAP42 (Di Como and Arndt, 1996). However, the functional significance of this association and the mechanism by which TAP42 controls translation initiation are unknown. TAP42 may alter the activity or specificity of the phosphatases, or the phosphatases may regulate an as yet unknown TAP42 activity. Our observation that NPR1 remains phosphorylated in the dominant rapamycin-resistant *tap42-11* mutant suggests that TAP42 may control NPR1 phosphorylation by negatively regulating phosphatases. Consistent with this model is the observation that rapamycin stimulates type 2A phosphatase activity in mammalian cells (Begum and Ragolia, 1996). Alternatively, TAP42 could be regulating NPR1 more indirectly and activating phosphatases, or even regulating NPR1 independently of phosphatases.

Table I. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype
JK9-3d**a**/α	*MAT**a**/MATα leu2-3,112/leu2-3,112 ura3-53/ura3-52 trp1/trp1 his4/his4 rme1/rme1 HML**a**/HML**a***
JK9-3d**a**	*MAT**a** leu2-3,112 ura3-52 trp1 his4 rme1 HML**a***
JK9-3dα	*MATα leu2-3,112 ura3-52 trp1 his4 rme1 HML**a***
JH12-17b	JK9-3d**a** *TOR2-1*
JH11-1c	JK9-3d**a** *TOR1-1*
JH18-3b	JK9-3d**a** *TOR1-1 TOR2-1*
MH349-3b	JK9-3dα *tor1::LEU2*
AS63-1d	JK9-3dα *tor1::LEU2 TOR2-1*
TK54-1b	JK9-3d**a** *npr1::URA3*
AS60	JK9-3d**a**/α *npr1::URA3/NPR1 tor1::LEU2/TOR1*
MH339	JK9-3d**a**/α *TRP1/TRP1 HIS4/HIS4 LEU2/LEU2*
TK109	JK9-3d**a**/α *npr1::URA3/npr1::URA3*
AS42-4b	JK9-3dα *TOR2-1 TRP1 HIS4 LEU2*
TS19-4c	JK9-3d**a** *tap42::kanMX*/YCplac111::*tap42-11*^{ts}

How is NPR1 controlling the degradation of TAT2? Although we have been unable to detect kinase activity with phosphorylated or unphosphorylated NPR1 and various potential substrates (unpublished results), our data suggest that NPR1 becomes active when dephosphorylated, and this activated form of NPR1 then signals the ubiquitination and degradation of TAT2, possibly by phosphorylating TAT2 directly. Phosphorylation of a cytoplasmic domain of a membrane protein is, in several cases, a prerequisite for ubiquitination and degradation (Hicke and Riezman, 1996; Hicke, 1997; Marchal *et al*., 1998). TAT2 is ubiquitinated upon starvation (Beck *et al*., 1998), and preliminary evidence indicates that TAT2 is also phosphorylated *in vivo*. The above predicts that TAT2 should be stable in a starved or rapamycin-treated *npr1* mutant. However, contrary to this prediction, we have found that TAT2 is still degraded in an *npr1* mutant (unpublished results). This may be because other kinases substitute for NPR1 in mediating the degradation of TAT2. The rapamycin- and FK506-resistant growth that we observe with an *npr1* mutant could be due to a weak stabilization that results in enough TAT2 to allow growth after several days of incubation on drug media but not enough to allow direct detection of higher TAT2 levels.

The general amino acid permease GAP1 and the specific amino acid permease TAT2 are regulated inversely. Our findings and the observation that NPR1 is required for GAP1 stability (Grenson, 1983a,b) suggest that the inverse regulation of the two types of permeases is mediated by NPR1. Under good nutrient conditions when NPR1 is phosphorylated and inactivated, GAP1 is degraded (Hein *et al*., 1995; Springael and Andre, 1998) and TAT2 is stable, whereas, under poor nutrient conditions when NPR1 is dephosphorylated and activated, GAP1 is protected and TAT2 is degraded. NPR1 may protect GAP1 and degrade TAT2 by phosphorylating both permeases directly. It will be of interest to determine if TOR and TAP42 are required for GAP1 degradation, as they are required for TAT2 stability.

There are many analogies between our findings and the role of mTOR/FRAP/RAFT1 in mammalian cells. Like TOR, mTOR also controls cell growth by sending both positive and negative signals. mTOR activates cell growth by phosphorylating and activating p70 s6 kinase, and inhibits a growth arrest programme by phosphorylating and inactivating the translational inhibitor 4E-BP1/PHAS-I (Thomas and Hall, 1997). Furthermore, although mTOR can phosphorylate both p70^{s6k} and 4E-BP1 *in vitro* (Brunn *et al*., 1997; Burnett *et al*., 1998), the phosphorylation of p70^{s6k} and 4E-BP1 by mTOR in cells may not be direct (Pullen and Thomas, 1997; Thomas and Hall, 1997; Burnett *et al*., 1998), and may involve intermediate components such as TAP42 and phosphatases (Murata *et al*., 1997; Pullen and Thomas, 1997; Chen *et al*., 1998). The mechanism by which mTOR signals to p70^{s6k} and 4E-BP1 may be similar to that by which TOR signals to NPR1. Also analogous and of interest is the finding that the TOR-related kinases ATM and DNA-PK may protect the tumour suppressor p53 from ubiquitin-dependent degradation (Keith and Schreiber, 1995; Lane, 1998).

Materials and methods

Strains and media

Saccharomyces cerevisiae strains used in this work are listed in Table I. All strains were isogenic JK9-3d derivates. The composition of rich medium (YPD) and synthetic minimal medium (SD) complemented with the appropriate nutrients for plasmid maintenance was as described (Guthrie and Fink, 1991). Ammonium and proline media, or media without ammonium or proline as a nitrogen source were prepared using yeast nitrogen base without ammonium sulfate and amino acids, supplemented with 2% glucose, tryptophan, histidine, leucine, and either 10 mM ammonium sulfate, 1 mg/ml proline, or no ammonium or proline. Rapamycin (gift of Sandoz Pharmaceutical) was used at a final concentration of 200 ng/ml. A 1 mg/ml stock solution of rapamycin was prepared in the drug vehicle 90% ethanol, 10% Tween. Rapamycin-untreated control cells were, in all cases, treated with drug vehicle alone. Resistance to FK506 (gift of Sandoz Pharmaceutical) was examined at concentrations of 50 μg/ml in YPD at 30°C. Strains examined for FK506 resistance were homozygous diploids as only diploids are sensitive to this drug (Heitman *et al*., 1991b).

Plasmids

pNPR1 (pJK20) is pSEY8 (2μ, *URA3*) containing *NPR1*. pHA-TAT2 (pAS55) is YEplac195 (2μ, *URA3*) (Gietz and Sugino, 1988) expressing functional N-terminally HA-tagged TAT2 under the control of its own promoter (Beck *et al*., 1998). pHA-NPR1 (pAS103) is YEplac195 (2μ, *URA3*) expressing functional HA-tagged NPR1 under the control of its own promoter. pHA-NPR1 was constructed in two steps, as follows. First, a 2.2 kb PCR-generated *Eco*RI–*Xba*I fragment containing the *NPR1* initiation codon and upstream region was cloned into YEplac195. The PCR primer containing the initiation codon and the *Xba*I site was 5′-CCTCTAGA**CAT**AATGTTTCGTAGAGCTTTCCT-3′ (*Xba*I site underlined and antisense initiation codon in bold). Secondly, a 2.6 kb PCR-generated *Xba*I fragment containing the *HA-NPR1* open reading frame was cloned into the plasmid derived from the first step. The PCR primers used for this second step were 5′-CCTCTAGA**TATCCATATG-ACGTTCCAGATTACGCT**TCTTCATTAACTCGATTGCTACAG-3′ (*Xba*I site underlined and *HA* open reading frame in bold) and 5′-CCTCTAGAAGTGGCGCGATATTTTAACGAAC-3′ (*Xba*I site underlined). The second primer corresponds to a sequence ~250 nucleotides downstream from the *NPR1* stop codon. The resulting sequence containing the *NPR1* initiation codon (bold), the *Xba*I site (underlined) and the *HA* open reading frame was 5′-CGAAACATT**ATG**TCTAGA**TAT-CCATATGACGTTCCAGATTACGCT**TCTTCATTA-3′.

Genetic techniques

Yeast plasmid DNA was isolated as described (Hoffman and Winston, 1987). Yeast transformation was perfomed by the lithium acetate procedure (Ito *et al*., 1983). *Escherichia coli* strain DH5α was used for propagation and isolation of plasmids as described (Sambrook *et al*., 1989).

DNA manipulations

Restriction enzyme digests and ligations were done by standard methods. All enzymes and buffers were obtained commercially (Boehringer Mannheim).

Disruption of NPR1

NPR1 was disrupted by replacing the internal 0.7 kb *Bgl*II fragment of the *NPR1* gene with a 1.0 kb *Bam*HI fragment containing the entire *URA3* gene. A 2.3 kb *Bam*HI fragment containing *npr1::URA3* was transformed into yeast. The integration was verified by Southern analysis.

Amino acid import studies

The rates of import of radiolabelled tryptophan were measured as described (Heitman *et al*., 1993). Cells were grown to early logarithmic phase in SD-URA medium and divided in two. Rapamycin was added to one of the two cultures at a final concentration of 200 ng/ml. The cultures were incubated for an additional 3 h at 30°C and then measured for amino acid uptake.

Preparation of cell extracts

Whole yeast cell extracts for SDS–PAGE and Western analysis were prepared by resuspending cells in 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM phenylmethyl sulfonyl fluoride (PMSF), lysing them with glass beads in a bead-beater, and removing cell debris by centrifugation at 500 *g* for 10 min at 4°C. Samples that were analysed for HA-TAT2 were denatured at 37°C for 10 min. Samples that were analysed for HA-NPR1 were denatured at 95°C for 5 min. SDS–PAGE and Western analysis were performed by standard methods using rat anti-HA antibody (3F10) from Boehringer Mannheim.

Alkaline phosphatase treatment of protein extracts

Approximately 50 μg of whole yeast cell extract were incubated with 1 U of alkaline phosphatase (Boehringer Mannheim) in the presence of 2 mM $MgCl_2$ for 30 min at 37°C. Samples were denatured at 95°C for 5 min and subjected to SDS–PAGE and Western analysis.

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