**PBS2**, a yeast gene encoding a putative protein kinase, interacts with the RAS2 pathway and affects osmotic sensitivity of *Saccharomyces cerevisiae*

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The yeast gene **PBS2** encodes a presumed protein kinase. The gene is essential for manifestation of resistance to the antibiotic polymyxin B. Deletion of **PBS2** enables a *ras2-530* null mutant to grow on nonfermentable carbon sources; overexpression of **PBS2** enhances viability of a *RAS2*Δ1 mutant. Overexpression of **PBS2** also diminishes cellular response to mating pheromone MFα. These results suggest that the **PBS2** and **RAS2** genes affect a common pathway that may communicate with the pheromone response pathway. In addition, disruption of **PBS2** renders cells sensitive to high osmolarity: exposure to 0.9 M-NaCl causes growth arrest, appearance of bizarre morphological forms, and eventual death. A mutation suppressing **pbs2** deletion has been found. That mutation restores full polymyxin B resistance but only partially corrects the osmotic sensitivity defect. These observations indicate that **PBS2** is involved in diverse physiological pathways in yeast.

Introduction

Polymyxin B, a complex antibiotic produced by cells of *Bacillus polymyxa*, is very toxic to bacterial, fungal and animal cells. The drug exerts its effects by damaging the integrity of cell membranes (Storm *et al.*, 1977), and at high concentration it causes cell lysis.

Polymyxin B has several important uses in biological studies. First, high concentrations of the antibiotic, a positively charged molecule, displace calcium ions from anionic phospholipids and cause disorganization of cell membranes (Storm *et al.*, 1977). Thus, the topography of eukaryotic membranes, as well as the role of calcium ions in the maintenance of membrane structure and activity, can be elucidated with the help of this antibiotic (Bearer & Friend, 1980; Kubesch *et al.*, 1987). Second, the drug produces interesting pharmacological effects. For example, it is one of the most potent neuromuscular blocking agents known (Singh *et al.*, 1980), and it promotes the conversion of membrane acetylcholine receptors from the resting activatable state to the desensitized state (Brazil *et al.*, 1989; Brown & Taylor, 1983). Third, the antibiotic inhibits Ca2+-activated K+ channels in mouse skeletal muscle (Weik & Lommen-donker, 1990) and in human red blood cells (Varecka *et al.*, 1987). In *Saccharomyces cerevisiae* polymyxin B is a powerful inhibitor of mating (Boguslawski, 1986).

Studies on polymyxin B resistance in yeast have revealed the existence of two genes, **PBS1** and **PBS2** (polymyxin B sensitivity), whose activities are essential for the resistance phenotype (Boguslawski, 1985; Boguslawski & Polazzi, 1987). The deduced amino acid sequence of the **PBS2** gene product bears a strong resemblance to polypeptides of the serine/threonine protein kinase family (Boguslawski & Polazzi, 1987; Hanks *et al.*, 1988), making it likely that the gene does encode a protein kinase. The *pbs1* mutation confers a high level of resistance to polymyxin B on yeast cells, but only if an intact **PBS2** gene is present (Boguslawski & Polazzi, 1987). Disruption of this gene results in a drug-sensitive phenotype. Conversely, overexpression of **PBS2** makes cells resistant to the drug even in the wild-type background.

The results discussed in the present communication show that, in addition to abolishing polymyxin B resistance, disruptions in the **PBS2** gene can suppress a recessive null mutation of the yeast **RAS2** gene, and that overexpression of **PBS2** attenuates the effect of a dominant **RAS2**Δ1 mutation. Furthermore, enhanced expression of **PBS2** diminishes the ability of cells to

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respond to a mating pheromone. These observations suggest that PBS2 may be a part of the signal transduction circuitry in yeast.

**Methods**

*Strains.* All yeast strains and plasmids used in this work are listed in Table 1. The media, growth conditions, and methods of genetic manipulation have been described by Sherman *et al.* (1983). YEPD medium contains 2% (w/v) glucose, 2% (w/v) Bacto peptone and 1% (w/v) yeast extract. Solid YEPD medium includes 2% (w/v) agar. All strains described here grow very well on this medium at 30°C.

*Pheromone sensitivity assay.* Approximately 10³ MATα cells were suspended in 3 ml of 0.7% agar in water and poured on synthetic minimal medium plates containing the necessary nutrients. After the agar overlay had solidified, sterile filter disks (6 mm diameter) were placed on the surface, and synthetic mating pheromone (MFα, Sigma) was applied to each disk. The plates were incubated at 30°C for 2-3 days until the zones of inhibition were easily observed.

*Viability assays.* The tests were performed essentially as described by Toda *et al.* (1985). Cells were grown at 30°C to stationary phase in YNN27 source or the nutrients) at 1 x 10⁶ cells ml⁻¹ (Toda *et al.*, 1985). After 96 h of shaking at 300 r.p.m. at 30°C, aliquots were plated on solid YEPD, and viability was determined by counting colonies on each plate after 2 d.

*Heat shock.* Cells were patched on minimal medium plates supplemented with the necessary nutrients and grown to confluence. The plates were sealed with Parafilm and floated in a 55°C water bath. After incubation, the plates were replicated onto YEPD and incubated at 30°C for 24 h.

*Osmotic sensitivity.* Cells were grown in patches on solid YEPD and replica-printed onto YEPD containing 0.9 M NaCl. The plate was incubated at 30°C for 72 h and photographed. Survival of the cells was tested by incubating the patches on the 0.9 M NaCl plate for 9 d and printing onto fresh YEPD.

*Phase-contrast and fluorescence microscopy.* Methods described by Adams & Pringle (1991) were used to assess the effect of high salt concentration on cell morphology. Cells were scraped from a plate of YEPD containing 0.9 M NaCl, washed, fixed in formaldehyde, and examined microscopically. Rhodamine–phalloidin conjugate (Molecular Probes) was used to determine actin distribution.

*Plasmid constructions.* Two integrating plasmids were used in this study (Fig. 1). Plasmid YIp5-AN117 carries a 1.17 kb internal fragment (AatII–NcoI) of the PBS2 gene in the YIp5 vector (Boguslawski & Polazzi, 1987). To create a disruption, pbs2-1, the plasmid was digested with the restriction endonuclease ClaI. The linear molecule was integrated into the chromosomal site, disrupting the open reading frame at amino acid 141. Alternatively, a deletion, pbs2-2, was created by substituting the Tcr-URA3+ portion of YIp5 (as a 3.2 kb ClaI–XmnI fragment) for the 1.26 kb ClaI–EcoRV portion of the open reading frame of the gene. The resulting plasmid pGB5 was digested with AatII, and the 4-kb fragment was integrated into the chromosome to create pbs2-2 with the protein coding sequence truncated at amino acid 38. Southern analysis confirmed the nature of integration in each case (data not shown), and the two constructions displayed essentially identical phenotypes in the tests described in this communication.

*For studies requiring overexpression of PBS2*, an episomal plasmid YEp24.PBS2 was constructed. For this purpose, the parent plasmid RSH (Boguslawski & Polazzi, 1987) was digested with SacI restriction endonuclease, the ends made flush with T4 phage DNA polymerase, and a SacI linker added. After ligation and transformation of Escherichia coli, the plasmid was isolated, digested with DraI and SalI, and a 3-kb DraI–SalI fragment, containing the entire PBS2 gene, was isolated by gel electrophoresis. The purified fragment was ligated into the YEp24 vector that had been digested with PseII and SalI.

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**Table 1. Saccharomyces cerevisiae strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype or relevant markers</th>
<th>Source or reference</th>
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<tr>
<td>YNN27</td>
<td>MATα trp1-289 ura3-52</td>
<td>R. W. Davis</td>
</tr>
<tr>
<td>GBH21</td>
<td>MATα trp1-289 ura3-52, pbs1-273</td>
<td>Boguslawski (1985)</td>
</tr>
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<td>GBH37</td>
<td>MATα trp1-289 ura3-52, pbs1-273, pbs2-2</td>
<td>This work, from GBH21</td>
</tr>
<tr>
<td>GBH38</td>
<td>MATα trp1-289 ura3-52, pbs1-273, pbs2-2</td>
<td>This work, from YNN27</td>
</tr>
<tr>
<td>GBH46</td>
<td>MATα trp1-289 ura3-52, pbs1-273, pbs2-2 PBS*</td>
<td>This work, from GBH37</td>
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<td>JC482</td>
<td>MATα his4 ura3 leu2</td>
<td>Cannon <em>et al.</em> (1986)</td>
</tr>
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<td>JC302</td>
<td>MATα ras2-530::LEU2+ his4 ura3 leu2</td>
<td>Cannon <em>et al.</em> (1986)</td>
</tr>
<tr>
<td>GBH74</td>
<td>MATα ras2-530::LEU2+ his4 ura3 leu2 leu2-1</td>
<td>This work, from JC302-26B</td>
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<tr>
<td>JC303-58</td>
<td>MATα ras2-530::LEU2+ his4 ura3 leu2 yak1(SRA7-14)</td>
<td>Cannon <em>et al.</em> (1986)</td>
</tr>
<tr>
<td>X10</td>
<td>MATα ras2-530::LEU2+ his4 ura3 leu2 leu2 yak1(SRA7-14) pbs2-1</td>
<td>This work, from JC303-58</td>
</tr>
<tr>
<td>SP1</td>
<td>MATα leu2 his3 ade8 ura3 trp1 can1</td>
<td>Toda <em>et al.</em> (1985)</td>
</tr>
<tr>
<td>GBH83</td>
<td>MATα leu2 his3 ade8 ura3 trp1 can1 pbs2-2</td>
<td>This work, from SP1</td>
</tr>
<tr>
<td>TK161-R2V</td>
<td>MATα leu2 his3 ade8 ura3 trp1 can1 RAS2α19</td>
<td>Toda <em>et al.</em> (1985), from SP1</td>
</tr>
<tr>
<td>GBH65</td>
<td>MATα leu2 his3 ade8 ura3 trp1 can1 RAS2α19 pbs2-2</td>
<td>This work, from TK161-R2V</td>
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</table>

* Strain GBH46 carries a mutation that restores polymyxin B resistance (PBS). The nature of this mutation has not been elucidated (see text for details).
Suppression of RAS2 mutations

Fig. 1. Integrating plasmids YIp5-AN117 and pGB5. The details of plasmid construction are described in Methods. The PBS2 sequences used to promote chromosomal integration are shown as filled arrows.

Fig. 2. Reduction of sensitivity to mating pheromone \( \alpha \) by overexpression of PBS2\(^+\). Increasing amounts of the synthetic pheromone MF\(_3\) were applied to each filter disk (disk 1, 1.8 \( \mu \)g; 2, 3.6 \( \mu \)g; 3, 5.4 \( \mu \)g; 4, 7.2 \( \mu \)g; 5, 10.8 \( \mu \)g). The plates were incubated for 2 d at 30 °C. A, GBH83, pbs2-2; B, SP1 transformed with YEp24.PBS2; C, SP1 transformed with YEp24 vector.

Fig. 3. Suppression of the ras2-530 phenotype by disruption of the PBS2 gene. Cells were grown at 30 °C on synthetic minimal medium plates with the necessary nutrients and replica-printed onto YPE medium (3% ethanol, 2% Bacto peptone, 1% yeast extract, 2% agar). The strains tested were as follows: A, JC482, wild-type; B, JC302-26B, ras2-530; C, GBH74, ras2-530 pbs2-1; D, JC303-58, ras2-530 yak1/SRA7; E, X10, ras2-530 yak1/SRA7 pbs2-1.

Results

Previous studies revealed that polymyxin B is a potent inhibitor of mating in *Saccharomyces cerevisiae* (Boguslawski, 1986). This observation suggested that genes involved in polymyxin B resistance may interact with some elements of signal transduction pathways in yeast. As shown in Fig. 2, overexpression of PBS2\(^+\) considerably reduces sensitivity of \( \alpha \) cells to the mating pheromone \( \alpha \) (disruption of PBS2 may slightly enhance sensitivity). Therefore, a component of the yeast pheromone signalling pathway may be influenced by PBS2-dependent phosphorylation. However, although PBS2 kinase belongs to the STE7 family of protein kinases (Hanks *et al.*, 1988), and the STE7 gene is essential for mating in yeast (Teague *et al.*, 1986), neither disruption nor overexpression of PBS2 can restore mating ability to cells lacking a functional STE7 gene (data not shown). Variation in copy number of PBS2 also has no effect on the activity of FUS1 gene (Truehart *et al.*, 1987; data not shown).

An important subset of signal transduction pathways is known to be regulated by two RAS genes which act as
modulators of adenyl cyclase activity (Field et al., 1990b; Toda et al., 1985). Mutations in the RAS2 gene confer pleiotropic effects on cells (Cannon et al., 1986; Toda et al., 1985). These effects include a decrease in cyclic AMP (cAMP) levels, enhanced sporulation in rich media, and accumulation of storage carbohydrates such as glycogen. In addition, ras2 mutants are unable to use nonfermentable carbon sources such as ethanol for growth (Cannon et al., 1986). However, these mutants grow very well on rich media such as YEPD that contain glucose as carbon source.

Disruption of the PBS2 gene in the strain bearing a null allele of the RAS2 gene (ras2-530 : LEU2) restores the ability of cells to grow on 3% ethanol (Fig. 3). However, the growth is not as robust as when a ras2 suppressor mutation in another protein kinase gene yak1 (Garrett & Broach, 1989) is present; this gene has been described as SRA7 by Cannon et al. (1986). In contrast to yak1/SRA7, pbs2 at 37 °C cannot suppress a cyr1/cdc35 (adenyl cyclase) temperature-sensitive defect (cyr1-2 and cdc35-10 alleles were tested) or a ras1 ras2 double mutation (not shown). The disruption neither enhances nor diminishes growth of the two cyr1 mutants at room temperature. Although these negative results may be explained by the weak suppressive effect of pbs2 (Fig. 3), they do suggest a possibility that PBS2 acts downstream of or in parallel to RAS2 but not downstream of CYR1; they also indicate that the targets for yak1 and pbs2 suppression are probably not related.

A dominant missense mutation that substitutes valine for glycine at position 19 (RAS2<sup>V<sub>d19</sub></sup>, activated RAS2) results in an increased level of cAMP in the cells, poor sporulation, lack of carbohydrate accumulation, and loss of viability upon starvation or heating (Toda et al., 1985).
affected by the condition of the PBS2 allele (Table 2, unpublished data). In contrast, most cells of the activated RAS2 mutant (strain TK161-R2V, quite unhealthy to begin with) died upon starvation. The survival of this strain was further depressed about fivefold by the pbs2 deletion but was enhanced about threefold by overexpression of PBS2+ (Table 2). These results are reinforced by the observation that pbs2 disruption enhanced heat-shock sensitivity, whilst overexpression of PBS2+ protected the cells from thermal death (Fig. 4).

Accumulation of glycogen by the various strains used in this study was examined by iodine staining (Cannon et al., 1986). There was no effect either of overexpression of PBS2+ or of pbs2 disruption or deletion on the staining, and the pattern of glycogen accumulation followed that of the corresponding parent. Thus, regardless of the status of the PBS2 allele, ras2-530 colonies became dark brown when exposed to iodine vapour, while the RAS2Δ110 colonies remained straw yellow (data not shown). As with adenyl cyclase mutants (cyr1), the lack of effect may be related to the weak suppression (perhaps because the threshold of sensitivity is different for growth on ethanol and carbohydrate accumulation). Nevertheless, PBS2 suppression, or lack of it, provides an additional means of discriminating between the several cellular effects of the RAS2 gene.

Polymyxin B is a membrane-active antibiotic, and genes controlling cellular responses to the drug may be logically thought to participate in membrane functions. One manifestation of membrane activity is osmoregulation or the ability of cells to cope with the increased salt concentration in the growth medium. As shown in Fig. 5, the wild-type cells and pbs1 (polymyxin B resistant) mutant grew well when 0.9 M-NaCl was present. In contrast, a pbs2 mutant and pbs1 pbs2 double mutants were unable to grow under such conditions (Fig. 5, C and D), and continued incubation resulted in death of these cells. Microscopic examination of cells exposed to 0.9 M-NaCl revealed (Fig. 6) that pbs2 mutant cells became morphologically aberrant, unable to form buds, but instead producing elongated, almost hypha-like extensions. However, as in normal cells, actin appeared to accumulate in the growing tip (Fig. 6, E). Cells grown in the absence of NaCl displayed normal morphology and viability (not shown). Interestingly, a strain selected as a polymyxin B resistant derivative of pbs1 pbs2 (i.e. bearing a suppressor of pbs2 disruption) showed only weak growth on 0.9 M-NaCl (Fig. 5, E), even though its level of resistance to polymyxin B equalled that of the pbs1 parent. The suppressor strain, although clearly inhibited by the high salt concentration, was morphologically normal (data not shown). These results may be interpreted as meaning that the aberrant morphology is only partially related to osmotic sensitivity of the pbs2 disruptant, and that involvement of PBS2 kinase in antibiotic resistance could be separated from its role in osmoregulation. The above observations, coupled with suppression of RAS2 mutations, suggest that PBS2 is involved in several diverse physiological processes, all of which may relate to cell membrane function.

Table 2. Viability of several yeast strains upon starvation in liquid medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP1</td>
<td>Wild-type</td>
<td>95.3</td>
</tr>
<tr>
<td>TK161-R2V</td>
<td>RAS2Δ110</td>
<td>0.37</td>
</tr>
<tr>
<td>TK161-R2V(YEp24.PBS2)</td>
<td>RAS2Δ110 PBS2++</td>
<td>1.20</td>
</tr>
<tr>
<td>GBH65</td>
<td>RAS2Δ110 pbs2-2</td>
<td>0.07</td>
</tr>
</tbody>
</table>

* PBS2+ is overexpressed in cells transformed with YEp24.PBS2 plasmid.
Discussion

The results of this study indicate that the RAS2 and PBS2 genes may be involved in a common pathway. The fact that deletions of PBS2 suppress the null ras2 phenotype of JC302-26B suggests that PBS2 acts (negatively) on an element downstream of RAS2; it also implies an antagonism between the products of the two genes. In addition, overexpression of PBS2 weakens the effect of the enhanced RAS2 activity of TK161-R2V, thus confirming the antagonistic relationship between PBS2 and RAS2. It is possible that RAS2 is a negative regulator of PBS2 activity; another possibility is that PBS2 overcomes the negative effect of PBS2 on a component downstream from these two genes (for example, by promoting dephosphorylation of that component). Alternatively or simultaneously, deletion of PBS2 may allow derepression of RAS1 gene that is normally repressed in the presence of nonfermentable carbon sources (Breviario et al., 1986), thus permitting growth of the ras2 PBS2 double mutant on ethanol. It is important to note that the suppression is not very strong, and that neither overexpression nor deletion of PBS2 allows cells to bypass other RAS functions or to restore a completely wild-type phenotype.

One cellular component directly downstream from RAS2 is the adenyl cyclase complex (encoded by CYRI, CAP, IRA1, and perhaps other genes; Fedor-Chaiken et al., 1990; Field et al., 1990a; Mitts et al., 1992, Vojtek et al., 1991). The RAS proteins control the activity of this complex (Fedor-Chaiken et al., 1990; Field et al., 1990a; Toda et al., 1985; Vojtek et al., 1991). Since PBS2 deletion or overexpression cannot suppress mutations either in the CYRI (CDC35) gene or in a double mutant ras1 ras2 (data not shown), changes in PBS2 do not allow cells to bypass the cyclase step. Nevertheless, a specific interaction can be envisioned. It is possible that the antagonistic relationship between RAS2 and PBS2 is reflected, in part, in the phosphorylation–dephosphorylation state of the adenyl cyclase complex: PBS2 kinase may down-modulate the complex activity, while RAS2 could promote dephosphorylation. If so, in a ras2 background, deletion of PBS2 should decrease the amount of a phosphorylated complex, thereby enhancing its basal activity, and thus permitting the ras2 mutant to grow on ethanol (Fig. 3). In RAS2\(^{\text{W19}}\) cells, which constitutive activation of the adenyl cyclase is the presumed reason for loss of viability upon starvation (Toda et al., 1985), diminished phosphorylation would lead to an increased cyclase activity (already enhanced by the activated RAS2 protein) and further depression of viability. Conversely, overexpression of PBS2 kinase would be expected to enhance cell survival (Table 2, Fig. 4), because it would counteract the overstimulation of the complex by the RAS2\(^{\text{W19}}\) protein.

At least two additional cellular elements could be the targets for the RAS2–PBS2 pathway or for PBS2 alone. First, as shown earlier (Fig. 2), overexpression of PBS2\(^{+}\) diminishes mating factor sensitivity. It may be significant that the pheromone response pathway in yeast is mediated by G proteins that share some structural and biochemical features with RAS proteins (Blumer & Thorner, 1991). An independent suggestion of ‘cross-talk’ between the pheromone response elements and the RAS–adenyl cyclase pathway has been recently presented by Arkingstall et al. (1991).

Second, as already pointed out, PBS2 is essential for the expression of polymyxin B resistance of pbsl mutants (Boguslawski & Polazzi, 1987). Thus, it is likely that the PBS1 gene product is another substrate for PBS2 kinase, and that resistance requires phosphorylation of the PBS1 product. However, other interpretations are possible. For example, it could be that the mutation in PBS1 results in an increase in PBS2 expression and, thus, resistance. Two pieces of evidence argue against this possibility. First, there is no difference in the amount of PBS2 mRNA detectable in either wild-type or pbsl cells (data not shown), and previous results indicate that at least four copies of intact PBS2\(^{+}\) are necessary to confer polymyxin B resistance in the wild-type, PBS1\(^{+}\), background (Boguslawski & Polazzi, 1987). Therefore, at least fourfold (and probably higher) derepression of PBS2 would have to result from pbsl mutation. Second, the resistance due to overexpression of PBS2\(^{+}\) is not observed at 37 °C, whilst that due to pbsl mutation is equally strong at 25 °C and 37 °C (not shown). This means that the biochemical basis for resistance must be different in these two cases even though PBS2 kinase (single gene copy) is essential for expression of the pbsl-conferred resistant phenotype.

pbsl mutants are deficient in net potassium uptake (R. Gaber and G. Boguslawski, unpublished data). This observation opens an attractive (but speculative) prospect that PBS1 encodes a (component of) a potassium channel that is dependent on the activities of the RAS2 and PBS2 genes. Such a possibility is supported by the evidence that animal RAS and other G proteins are linked to eukaryotic potassium channel activity (Rudy, 1988), and that polymyxin B inhibits calcium-activated potassium channels in mammalian cells (Varecka et al., 1987; Weik & Lonendonker, 1990). A very recent report (Schultz et al., 1992) demonstrates that potassium flux regulates adenyl cyclase activity in Paramecium. The cloning and characterization of PBS1 will be necessary to determine
whether and how the \textit{RAS2} gene product might interact with potassium channels and with the molecules involved in polymyxin B resistance in yeast.

\textbf{Addendum.} While this work was in progress, I was informed by Dr Richard Kahn of the National Cancer Institute, Bethesda, MD, USA, that the previously reported sequence of \textit{PBS2} (Boguslawski \& Polazzi, 1987) is in error. The corrections are as follows: nucleotide 665 (C) is replaced by a G, and nucleotide 667 (G) is replaced by a C. These replacements change amino acids Ala222 and Val223 to Gly222 and Leu223, respectively. Further, an additional T residue is found in the nucleotide sequence at the position 2002 that results in a replacement of Tyr668 by Leu668 and in the appearance of a nonsense codon TAG immediately after the Leu668 residue. Therefore, the predicted \textit{PBS2} polypeptide contains 668 amino acids rather than the previously calculated 710 amino acids. The changes in the sequence have no bearing on the previous and current genetic data or on the surmised membership of the \textit{PBS2} product in the protein kinase family.

This paper is dedicated to the memory of my mother.

I thank Richard Gaber for his thoughtful comments on the manuscript and Mark Goebel for allowing me to use his fluorescence microscope.

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