A Proteasome-regulated Glycogen Synthase Kinase-3 Modulates Disease Response in Plants*\(^{\text{S}}\)

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Glycogen synthase kinase-3 (GSK-3)\(^{1}\) is a key player in various important signaling pathways in animals. The activity of GSK-3 is known to be modulated by protein phosphorylation and differential complex formation. However, little information is available regarding the function and regulation of plant GSK-3/shaggy-like kinases (GSKs). Analysis of the in vivo kinase activity of MsK1, a GSK from *Medicago sativa*, revealed that MsK1 is active in healthy plants and that MsK1 activity is down-regulated by the elicitor cellulase in a time- and dose-dependent manner. Surprisingly, cellulase treatment triggered the degradation of the MsK1 protein in a proteasome-dependent manner suggesting a novel mechanism of GSK-3 regulation. Inhibition of MsK1 kinase activity and degradation of the protein were two successive processes that could be uncoupled. In a transgenic manner. Surprisingly, cellulase treatment triggered the degradation of the MsK1 protein in a proteasome-dependent manner suggesting a novel mechanism of GSK-3 regulation. Inhibition of MsK1 kinase activity and degradation of the protein were two successive processes that could be uncoupled. In a transgenic approach, stimulus-induced inhibition of MsK1 was impeded by constant replenishment of MsK1 by a strong constitutive promoter. MsK1 overexpressing plants exhibited enhanced disease susceptibility to the virulent bacterial pathogen *Pseudomonas syringae*. MAP kinase activation in response to pathogen infection was compromised in plants with elevated MsK1 levels. These data strongly suggest that tight regulation of the plant GSK-3, MsK1, may be important for innate immunity to limit the severity of virulent bacterial infection.

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Glycogen synthase kinase-3 (GSK-3)\(^{2}\) was originally identified in mammals as a regulator of glycogen metabolism (1, 2). Despite its name, GSK-3 is a multifunctional serine/threonine protein kinase that acts as a regulator of numerous signaling pathways (3–5). As a component of the Wnt signaling pathway, GSK-3 participates in the regulation of cell proliferation, cell fate specification, and differentiation throughout the animal kingdom (6, 7). Misregulation of this pathway promotes degenerative diseases and cancer. GSK-3 is present in a multiprotein complex that can have variable constituents. In the absence of Wnt, GSK-3 phosphorylates the transcriptional regulator β-catenin and thereby targets β-catenin for degradation. When cells receive Wnt signals, the protein complex undergoes rearrangement (8–10) and GSK-3 activity is inhibited by an as yet not fully understood mechanism. As a consequence, β-catenin accumulates and modulates gene expression.

GSK-3 is also an integral part of the mammalian phosphatidylinositol 3-kinase pathway, which is involved in controlling cell survival, proliferation, apoptosis as well as metabolism. Upon stimulation of this pathway by insulin, growth factors, or stress, GSK-3 is phosphorylated by protein kinase B (11, 12). Biochemical and structural work suggest that the phosphorylated N terminus of GSK-3 prevents substrate binding and blocks access to the catalytic center (13–15). GSK-3 activity appears to be inhibited by this means in several animal signaling pathways (3).

GSK-3 is evolutionary conserved among eukaryotes. Mammals have two GSK-3 genes, whereas *Drosophila melanogaster* generates numerous isoforms from a single SHAGGY-locus by differential splicing. *Saccharomyces cerevisiae* encodes four GSK-3 homologs involved in mitotic chromosome segregation, meiosis, stress response, and metabolic regulation. Plants have a family of GSK-3/SHAGGY-like kinases (GSKs) that can be grouped into four classes according to sequence similarity (16, 17). Based on recent functional data plant GSKs appear to be involved in hormone signaling, development, and stress responses (16). RNA expression analyses in several plant species have shown that different GSKs display distinct, organ-specific developmental RNA expression patterns and respond differently to diverse stimuli (18–28).

Cloning of the brassinosteroid-insensitive mutant *bin2* (brassinosteroid-insensitive 2) from *Arabidopsis thaliana* revealed that *BIN2* encodes a GSK (29). *bin2* is allelic to *dwarf12* (30) and *iscu1* (31). Brassinosteroids (BRs) are plant steroid hormones that are important for cell growth and development. *BIN2* is a negative regulator of the *Arabidopsis* BR response. Genetic analyses place *BIN2* downstream of the plasma-membrane leucine-rich repeat receptor-like kinase *BRI1* (brassinosteroid-insensitive 1), which mediates the perception of BR signals (32, 33). Conceptually, *BIN2* phosphorylates and negatively regulates the BR-responsive transcription factors, BES1 (*BRI1*-EMS-suppressor 1) and BZR1 (brassinazole-resistant 1) (34–39). It has been proposed that BR signaling inhibits *BIN2* activity; however, to date no BR-dependent regulation of...
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BIN2 at the level of the transcript, the protein, or protein activity has been demonstrated. Recently, two additional BIN2-related GSKs have been implicated in BR signaling (39).

Some data point to a role for GSKs in plant stress responses. In Medicago sativa, WIG (wound-induced GSK) is activated upon wounding of leaves. Activation of WIG appears to be a post-translational process, whereas inactivation requires the transcription and translation of one or more proteins (20). MsK4 activity is rapidly induced in alfalfa roots by high salinity stress. Overexpression of MsK4 modulates stress tolerance and carbohydrate metabolism (40). In A. thaliana, plants overexpressing AtGSK-1 showed enhanced salt and drought tolerance (41).

Plant growth and development is severely affected by a multitude of adverse environmental conditions. To respond to these threats, plants have developed sophisticated signal transduction mechanisms that mediate and coordinate physiological responses. Although our knowledge about plant stress signaling has advanced, major gaps still remain. We aimed at identifying novel components in plant stress signal transduction. In a screen for stress-induced alteration of in vitro GSK kinase activities, we identified MsK1, an alfalfa GSK, as an elicitor-responsive GSK. Molecular and phenotypical analyses of transgenic plants indicate that MsK1 has a defense-related function in the plant innate immune response. Moreover, a novel molecular mechanism for GSK regulation is presented.

EXPERIMENTAL PROCEDURES

Plant Cultivation—M. sativa cv. Europa plants were grown hydroponically for 4 days. After treatment with the indicated concentrations of cellulase (Fluka) or proteasome inhibitor MG115 (Sigma), roots were detached beneath the hypocotyl and shock-frozen in liquid nitrogen.

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A. thaliana ecotype Columbia (Col-0) seeds were germinated on 0.5 × MS medium (Sigma). After 9 days, seedlings were transferred to soil. Plants were grown under long day conditions (16 h light/8 h dark) at 50 μE/m² s cool white light. For infection with Pseudomonas syringae, plants were grown under short day conditions (8 h light/16 h dark). For kinase assays and Western analysis, leaves were detached at the petioles and incubated for 2 h in 0.5 × MS medium plus 2% glucose prior to addition of cycloheximide (Sigma), cellulose, or treatment with P. syringae as indicated.

Generation of MsK1-Myc Overexpression Plants—The MsK1 cDNA was tagged with the Myc epitope and put under control of the Cauliflower Mosaic Virus 35 S-promotor and cloned into the binary plant expression vector pGreenII 0029 (42). This construct was used to transform A. thaliana Col-0 plants with the floral dipping method (43). Transformed plants were selected by germination on kanamycin-containing medium. Selected by germination on kanamycin-containing medium.

MsK1-specific Antibody—A rabbit polyclonal antibody was raised against the synthetic peptide QCPFLGL from the C-terminus of MsK1 (Davids Biotechnologie). Crude serum antibody was used for immunocomplex kinase assays. For Western analysis, peptide affinity-purified antibody was used. T3/T7 coupled in vitro translation kit (Amersham Biosciences) was used to in vitro translate MsK1, MsK2, MsK4, WIG, and SIMK. Immuno depletion assays were performed by preincubation of 2 μl of MsK1 antibody with 20 μg of immunization peptide at 4 °C prior to Western blotting.

Protein Extraction from Plant Material—Total proteins were extracted from A. thaliana leaves and M. sativa roots by grinding in homogenization buffer (25 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 15 mM EGTA, 75 mM NaCl, 1 mM dithiothreitol, 1 mM NaF, 0.5 mM NaVO₃, 5 mM β-glycerophosphate, 15 mM 4-nitrophenyl phosphate bis-[tris(hydroxymethyl)aminomethane], 0.1% Tween 20, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin) as described (44). Protein amounts were determined by the Bio-Rad Protein Assay (Bio-Rad) according to the manufacturer’s specifications.

Western Analysis—Equal amounts of proteins were separated by 10% SDS-PAGE and blotted to polyvinylidene difluoride membranes (Millipore). Membranes were probed with anti-MsK1 or anti-Myc (A-15, Santa-Cruz) antibodies. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Santa-Cruz) was used as a secondary antibody. The reaction was detected by enhanced chemiluminescence using the CDP-Star™ detection reagent (Amersham Biosciences).

Immunocomplex Kinase Assays—100 μg of total protein extracts were used for immunocomplex kinase assays as previously described (44). Depending on the batch, the substrate myelin basic protein (MBP; Sigma) was a single or a double band. MsK1-antibody was used to immunoprecipitate MsK1. MsK1-Myc was immunoprecipitated with Myc antibody, 9E10. Specific antibodies against AtMPK3 and AtMPK6 (45, 46) were used to determine MAP kinase activities.

P. syringae Growth Curve Assay—Virulent P. syringae pv. tomato DC3000 (Pst) or avirulent Pst DC3000 AvrRpt2+ was used in all experiments (47–49). Bacteria were grown on NYGA medium (0.5% trypton peptone, 0.3% yeast extract, 2% glycerol, 1.5% Bacto-agar) at 28 °C. For plant infection, liquid cultures were inoculated from plates and grown in NYG medium (0.5% trypton peptone, 0.3% yeast extract, 2% glycerol) at 28 °C overnight. Plants were vacuum infiltrated and bacterial titers were determined as described in Ref. 48.

RESULTS

MsK1 Activity and Protein Levels Are Regulated by Cellulase—To identify novel functions for plant GSKs, we transiently expressed alfalfa GSKs in protoplasts and analyzed their kinase activities in response to various treatments (50). MsK1 emerged as a potential stress-regulated signaling component. Previously, MsK1 was shown to be expressed in leaves, stems, flowers, and roots (19). To study the function of the MsK1 protein kinase in response to stress, we produced an antibody against MsK1 (supplemental Fig. 1) and used this antibody to determine the in vivo kinase activity of MsK1 by immunokinase assays after exposure of plants to various challenges. We found MsK1 activity to be high under normal growth conditions, whereas exposure to cellulase, which has been described as an elicitor of plant defense responses (46), caused reduced MsK1 activity in a dose- and time-dependent manner. Alfalfa seedlings were exposed to increasing concentrations of cellulase for 10 min. Protein extracts from roots of these seedlings were immunoprecipitated with the MsK1 antibody. Subsequent kinase assays using MBP as a substrate showed that exposure to 0.05% or higher concentrations of cellulase for 10 min resulted in a decrease of MsK1 activity that was dose-dependent.
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Concentrations of cellulase abated MsK1 kinase activity (Fig. 1A, upper panel). Protein gel blot analyses of the same cell extracts revealed that MsK1 protein levels also decreased with increasing cellulase concentrations concomitant with the appearance of a high molecular smear (Fig. 1A, middle panel). Staining of the membrane with Ponceau controlled for equal protein loading (Fig. 1A, lower panel). Cellulase treatment did not affect the steady state RNA expression level of MsK1 (data not shown).

To examine the kinetics of MsK1 down-regulation by cellulase, roots were treated with 0.1% cellulase and samples were taken at different time points. Immunokinase assays with the MsK1 antibody showed that MsK1 activity dropped as early as 30 s after application of cellulase and stayed low during the experimental period of 1 h (Fig. 1B, upper panel). In contrast to the rapid decline in MsK1 activity, MsK1 protein levels remained high for 5 min before decreasing (Fig. 1B, middle panel). The high molecular smear appeared after only 30 s of cellulase treatment. These data indicate that the inhibition of MsK1 activity precedes the degradation of the MsK1 protein.

Proteasome-dependent Regulation of MsK1—Proteasome-mediated protein degradation plays an important role in many signaling pathways (51–53). To test whether the disappearance of the MsK1 protein and the concomitant appearance of the high molecular smear depend on the 26 S proteasome, seedlings were pretreated with the proteasome inhibitor N-carbobenzoxy–Leu–Leu–norvalinal (MG115) prior to cellulase exposure. Immunokinase assays showed that MsK1 activity was comparable in untreated and MG115-treated roots. Application of cellulase decreased MsK1 activity irrespective of the proteasome-inhibitor pretreatment (Fig. 2, upper panel). However, in contrast to roots that had been directly challenged with cellulase, MsK1 protein levels of MG115-pretreated roots remained high (Fig. 2, middle panel). Taken together, inhibition of the proteasome with MG115 prevented MsK1 degradation but did not prevent inhibition of MsK1 kinase activity. These results indicate that MsK1 is controlled at two levels that can be uncoupled: MsK1 kinase activity is first blocked by cellulase and MsK1 protein is then subsequently degraded by a proteasome-dependent mechanism.

Post-transcriptional Regulation of Constitutively Overexpressed MsK1—To investigate the functional role of MsK1, transgenic plants with constitutively high levels of MsK1 activity were analyzed. We generated A. thaliana plants expressing a Myc-tagged version of MsK1 under the control of the strong Cauliflower Mosaic Virus 35 S-promoter. Several transgenic lines were obtained. Lines 3 and 13 carried a single T-DNA insertion and were thus selected for further characterization. Protein gel blot analysis using a Myc antibody showed that MsK1-Myc protein was present in lines 3 and 13. No signal was obtained in wild-type plants or in plants transformed with the empty vector (Fig. 3A, upper panel). Immunokinase assays using a Myc antibody revealed that MsK1-Myc kinase was active under normal growth conditions in lines 3 and 13 (Fig. 3A, lower panel).

To analyze the regulation MsK1 in Arabidopsis, MsK1 protein kinase activity was determined in MsK1 overexpressing plants of line 3 after cellulase treatment. Immunokinase assays with the Myc antibody revealed that MsK1-Myc kinase activity was high in untreated plants and was only marginally reduced by cellulase treatment (Fig. 3B, upper panel). Similarly, MsK1 steady state protein levels remained high in cellulase-treated plants (Fig. 3B, middle panel). In wild-type Medicago plants, MsK1 is rapidly inactivated and degraded when exposed to cellulase (Fig. 1). The constant supply of MsK1-Myc by the strong constitutive 35 S-promoter might mask the regulation of
MsK1-Myc in the transgenic Arabidopsis plants. Indeed, when protein synthesis was inhibited by cycloheximide (CHX) prior to application of cellulase, MsK1-Myc activity and protein levels decreased in response to cellulase (Fig. 3B). Similar results were obtained with transgenic line 13 (data not shown). Wild-type plants showed neither any Myc-kinase activity nor any protein recognized by the Myc antibody in protein gel blot analysis. These data suggest that MsK1 is regulated post-transcriptionally both in Arabidopsis and Medicago.

**MsK1 Overexpressing Plants Are More Sensitive to Virulent P. syringae**—The MsK1-Myc overexpressing plants were indistinguishable from wild-type and vector control plants when grown under standard conditions. Because cellulase can induce defense responses, the sensitivity of MsK1-Myc overexpressing plants to the bacterial pathogen *P. syringae* pv. tomato (Pst) DC3000 was assessed. Pst is an agriculturally important plant pathogen that is also strongly pathogenic toward *A. thaliana*. Pst encodes cell wall degrading enzymes implicated in virulence (54) and belongs to the γ subgroup of Proteobacteria that include a number of important animal and plant pathogens.

Five-week-old MsK1 overexpressing and control plants were vacuum infiltrated with $10^8$ colony forming units/ml of Pst DC3000. Samples were taken immediately after infiltration and 1, 2, 3, and 4 days post-infection and colony forming units/cm² leaf area were calculated for each line (Fig. 4A). Leaf disks were harvested from infected plants and bacterial titers calculated. Error bars indicate the standard deviation calculated from differences between 8 plants per line. The experiment was repeated independently 3 times with similar results. B, MsK1-Myc kinase activity and protein amount upon infection with Pst DC3000. Detached leaves from 5-week-old wild-type and MsK1-Myc overexpressing (line 3) plants were either pretreated with 100 μM CHX or directly vacuum infiltrated with Pst DC3000 at a concentration of $10^8$ colony forming units/ml in 10 mM MgCl₂ for 2 min. Control leaves (Ctrl) were infiltrated with 10 mM MgCl₂. Leaves infiltrated with CHX, but not infiltrated, were harvested together with seedlings 1 h after infiltration with Pst DC3000 or MgCl₂. MsK1-Myc kinase activity was measured at the indicated time points by immunocomplex kinase assays with Myc antibodies and MBP as a substrate. MsK1-Myc protein levels were detected by Western blot analysis with Myc antibodies. Ponceau staining after Western blotting is shown as a loading control (lower panel).

![Proteasome-targeted GSK-3 in Plant Disease Response](image-url)
MsK1 Modulates MAP Kinase Activation in Response to Pseudomonas Infection—MAP kinases are integral components of defense pathways (55, 56). The MAP kinases AtMPK3 and AtMPK6 are activated in response to elicitors (46, 57–59). To elucidate the MsK1 kinase activity and protein levels after infection with virulent Pseudomonas, leaves of wild-type and line 3 where either infiltrated with Pst DC3000 or with MgCl₂ as control (Fig. 4B). Neither MsK1-Myc kinase activity nor protein levels changed significantly upon bacterial infection. However, when protein synthesis was inhibited by CHX prior to infection with Pseudomonas, both MsK1-Myc kinase activity and protein levels dropped. Infiltration of CHX-incubated leaves with MgCl₂ did not change MsK1-Myc activity or protein amounts.

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DISCUSSION

Whereas GSK-3 is recognized as a crucial component of diverse cellular processes and much effort has been put into analyzing the molecular regulation of GSK-3 in animals, cellular functions of plant GSKs are just beginning to emerge and their regulation remains obscure. In this study, we discovered that cellulase inhibits the activity of MsK1. Interestingly, we found that following inhibition of MsK1 activity by cellulase, MsK1 protein is degraded in a proteasome-dependent manner. In a transgenic approach, we abrogated the reduction of MsK1 activity and protein levels by stress. These plants showed an enhanced pathogen susceptibility phenotype, indicating a defense-regulatory function for MsK1.

In natural environments, plants are constantly exposed to pathogens that can severely reduce host fitness. Plants have developed an array of constitutive and inducible defense strategies to combat pathogen attack (60). Preformed barriers including the plant cell wall effectively repel many pathogens. In a second line of defense, plants respond to pathogen invasion by a complex web of signaling pathways aimed at restricting pathogen growth. Significant progress has been made in understanding defense-related pathways in incompatible interactions between avirulent microbes and plants. However, little is known about the signaling mechanisms that restrict the growth of virulent pathogens in compatible interactions.

Here, we provide evidence that MsK1 might serve as a negative regulatory component involved in limiting the growth of the virulent bacterial pathogen Pseudomonas syringae. MsK1 kinase activity was found to be high in healthy plants but was inhibited following elicitor treatment. In transgenic plants, expressing MsK1 from a strong constitutive promoter MsK1 was not impaired by pathogen infection. However, when translation was blocked, infection with Pseudomonas led to inhibition of MsK1 activity and reduction of MsK1 protein levels, indicating that the constant replenishment of MsK1 by the constitutive promoter could override the pathogen-induced repression of MsK1. Overexpression of MsK1 allowed significantly more growth of Pst DC3000 as compared with wild-type plants, indicating a negative regulatory role of MsK1 in basal resistance. Basal resistance to invasive pathogens is an important defense mechanism as without it, plants become extremely susceptible to even mild infections.

Interestingly, MsK1 overexpressors showed enhanced susceptibility to virulent Pseudomonas but retained the ability to restrict growth of an isogenic avirulent strain carrying the avirulence gene AvrRpt2+. This observation indicates a role for MsK1 in the basal defense response rather than in 

Figure 5. Activation of AtMPK3 and AtMPK6 is reduced in MsK1-Myc overexpressors upon infection with virulent Pseudomonas. Leaves from 5-week-old wild-type, vector control, and MsK1-Myc overexpressing (line #3 and #13) plants were vacuum-infiltrated with Pst DC3000 at a concentration of 10^8 colony forming units/ml in 10 mM MgCl₂ for 2 min. MAPK activity was analyzed 15, 30, and 60 min after infiltration with Pseudomonas (+). Control leaves were infiltrated with MgCl₂ alone (−). AtMPK3 (A) and AtMPK6 (B) kinase activities were determined by immunocomplex kinase assays with AtMPK3 and AtMPK6 antibodies, respectively, using MBP as a substrate.

sors as compared with control plants. On day 4, both transgenic and control plants started to show symptoms of disease and bacterial titers decreased. When plants were infected with the virulent strain, Pst DC3000 AvrRpt2+, no difference was observed in the susceptibility between MsK1 overexpressing and control plants (supplemental Fig. 2).

To elucidate the MsK1 kinase activity and protein levels after infection with virulent Pseudomonas, leaves of wild-type and line 3 where either infiltrated with Pst DC3000 or with MgCl₂ as control (Fig. 4B). Neither MsK1-Myc kinase activity nor protein levels changed significantly upon bacterial infection. However, when protein synthesis was inhibited by CHX prior to infection with Pseudomonas, both MsK1-Myc kinase activity and protein levels dropped. Infiltration of CHX-incubated leaves with MgCl₂ did not change MsK1-Myc activity or protein amounts.

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Plant defense responses involve a complex interplay of plant hormones (64). Salicylic acid (SA) levels increase in response to bacterial infection, and plants that cannot accumulate SA are highly susceptible to infection with Pseudomonas. The enhanced disease susceptibility of MsK1 overexpressing plants could be due to a defect in SA accumulation. However, determination of SA levels following Pst infection showed normal accumulation of free SA and SA glycoside as compared with wild-type plants. Similarly, ethylene and jasmonic acid levels were comparable in wild-type and in MsK1 overexpressing plants (data not shown), indicating that the enhanced disease susceptibility of MsK1 overexpressors is not an effect of impaired hormone levels. These observations suggest that MsK1-based signaling functions independently or downstream of SA, ethylene, and jasmonic acid.

In this study, we show that AtMPK3 and AtMPK6 are activated by virulent Pst DC3000 and that full activation of AtMPK3 and AtMPK6 depends on MsK1. MAP kinases are integral elements of defense signaling pathways and mediate the activation of innate immune responses in plants and animals (55, 56, 65). In plants, AtMPK3 and AtMPK6 are activated in response to elicitors (46, 57–59) and silencing of AtMPK6 has been reported to compromise resistance to avirulent and virulent P. syringae (66). In the presence of constitutive high levels of MsK1 (that do not decrease after pathogen infection), we observed a reduced pathogen-induced activation of AtMPK3 and AtMPK6. The compromised activation of AtMPK3 and AtMPK6 by Pst DC3000 infection in MsK1 overexpressing plants positions MsK1 in the defense signaling network upstream of these MAP kinases.

Our results imply that MsK1 might act as a negative regulatory component in a signal transduction pathway important for basal immunity. Inhibition of MsK1 activity and the subsequent degradation of MsK1 by the proteasome appear to be important for restricting pathogen growth. Over the past few years, the proteasome has been recognized as a crucial regulator of plant responses to pathogens. SGT1 (suppressor of the G2 allele of photomorphogenic 9) signalosome are involved in protein degradation by the proteasome, which is a novel mechanism for GSK-3 regulation. Additionally, SGT1 has been recognized as a crucial regulator of plant immunity. GSK-3 is activated in response to different signals. Wounding transiently activates WIG (20) at the post-translational level and hyperosmotic stress induces MsK4 activity (40). Similarly, in Caenorhabditis elegans and Dictyostelium GSK-3 is activated in response to specific stimuli (72). Thus, distinct GSKs might serve as positive or negative regulators and the kinase activity could be induced or inhibited upon pathway stimulation, opening the possibility that GSK-3 signaling is highly complex in nature.

The molecular basis for innate immunity is remarkably conserved across kingdom borders (73). In this regard, it is interesting that GSK-3 was recently shown to be involved in modulating the innate immune system in mammals (74, 75). In response to pathogenic stimuli, GSK-3 exerts a critical function in differentially controlling the production of pro-versus anti-inflammatory cytokinins. However, whereas the regulatory function of GSK-3 in defense appears to be evolutionarily conserved between animals and plants, MsK1 seems to be regulated by a novel mechanism involving targeted protein degradation by the proteasome.

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REFERENCES
1. Embi, N., Rylatt, D. B., and Cohen, P. (1980) Eur. J. Biochem. 107, 519–527
2. Woodgett, J. R., and Cohen, P. (1984) Biochim. Biophys. Acta 788, 339–347
3. Cohen, P., and Frame, S. (2001) Nat. Rev. Mol. Cell Biol. 2, 769–776
