Wound-Induced Expression and Activation of WIG, a Novel Glycogen Synthase Kinase 3

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Glycogen synthase kinase 3 (GSK-3) is involved in the regulation of several physiological processes, including glycogen metabolism, protein synthesis, transcription factor activity, and developmental control. Although GSK-3–like genes have been isolated from plants, no function for any of these kinases has been defined. We report here that the alfalfa wound-induced gene (WIG, for wound-induced GSK-3), encoding a functional plant GSK-3–like kinase, is activated when the alfalfa leaves are wounded. Although WIG transcripts are hardly detectable in mature leaves, WIG mRNA accumulates rapidly after wounding. Using a peptide antibody that specifically recognizes p53WIG, we show that p53WIG kinase is activated immediately after wounding. Wound-induced activation of p53WIG kinase is a post-translational process, because the concentrations of p53WIG protein do not change in intact and wounded leaves, and inhibition of transcription or translation does not block activation by wounding. However, inactivation of p53WIG kinase, which usually occurs within 60 min after wounding, is dependent on transcription and translation of one or more protein factors. These data suggest that the WIG kinase is involved in wound signaling in plants.

INTRODUCTION

Glycogen synthase kinase 3 (GSK-3) was originally defined as a serine/threonine kinase that phosphorylates and either inactivates glycogen synthase or activates ATP-Mg-type 1 protein phosphatase (Plyte et al., 1992; Yang et al., 1992). Subsequently, GSK-3 has been shown to be able to phosphorylate several substrates, including the microtubule-associated protein tau; various transcription factors, such as c-Myc, c-j un, and c-Myb; and adenomatous polyposis coli (Mandelkow et al., 1992; Plyte et al., 1992; Rubinfeld et al., 1996). In mammals, GSK-3 is implicated in diverse aspects of Wnt and phosphatidylinositide 3-kinase signaling (Alessi and Cohen, 1998; Brown and Moon, 1998; Downward, 1998; Anderton, 1999). Genetic evidence indicates that the Drosophila GSK-3 homolog zeste-white3/shaggy is required at several developmental stages during segmentation and neurogenesis (Simpson et al., 1988; Perrimon and Smouse, 1989). In Xenopus, GSK-3 regulates ventral differentiation during early development (He et al., 1995). The Dictyostelium GSK-3 homolog GSKA has been found necessary for correct differentiation (Harwood et al., 1995). Genetic analysis in budding and fission yeast indicated that GSK-3s also function in controlling the cell division cycle. In Saccharomyces cerevisiae, the GSK-3 homologs MCK1 and MDS1 play a role in chromosomal segregation (Puziss et al., 1994), and the Schizosaccharomyces pombe GSK-3 homolog Skp1 regulates cytokinesis (Plyte et al., 1996). Recent results also established a role of GSK-3 in mammalian cell cycle regulation, demonstrating that GSK-3β regulates the stability of cyclin D1 (Diehl et al., 1998). In contrast to the two members of the GSK-3 family in mammals, plants appear to contain a much larger set of divergent genes encoding GSK-3–like enzymes (Pay et al., 1993; Bianchi et al., 1994; Decroocq-Ferrant et al., 1995; Einzenberger et al., 1995; Jonak et al., 1995; Dornelas et al., 1999). Except for complementation of the yeast MCK1 gene by Arabidopsis AtGSK-1 (Piao et al., 1999), only expression data are available for some of the other identified plant GSK-3–like genes (Pay et al., 1993; Decroocq-Ferrant et al., 1995; Einzenberger et al., 1995; Jonak et al., 1995; Tichtinsky et al., 1998; Dornelas et al., 1999), and no direct function for any of these genes has been defined. Here, we provide evidence that a novel member of the alfalfa GSK-3 family, WIG (for wound-induced GSK-3), is potentially involved in wound response signaling. We have observed that the WIG gene is specifically induced by wounding. More importantly, the WIG gene product, p53WIG kinase, is activated by wounding. Different lines of evidence indicate that p53WIG kinase is activated by a post-translational mechanism, but its inactivation is mediated
through transcription and translation of one or more protein factors.

**RESULTS**

**Wounding Induces the Transcription of WIG, an Alfalfa Gene Encoding a GSK-3-Type Protein Kinase**

While screening for new members of the GSK-3 family from alfalfa, we isolated a full-length cDNA that showed 51 and 56% identity to mammalian GSK-3α and GSK-3β, respectively (Figure 1). It was also ~50% identical to the Drosophila shaggy kinases. Comparisons with the known GSK-3s from plants showed that WIG shared extensive sequence similarity with three previously identified alfalfa GSK-3s: MsK1, MsK2, and MsK3 (Pay et al., 1993). However, the similarity was restricted to the respective kinase domains and did not extend into the N-terminal sequences. In contrast, WIG showed high sequence identity (75%) over the entire length of Arabidopsis ASKβ (Figure 1), suggesting that the N termini are conserved in different plant species and that WIG and ASKβ may be orthologs.

**Production of a WIG-Specific Antibody**

To study the function of the WIG protein kinase, we produced a peptide antibody against the C terminus of WIG. In crude protein extracts prepared from suspension-cultured alfalfa cells, which express high amounts of the WIG gene (data not shown), the affinity-purified antibody recognized a single protein of 53 kD, in good agreement with the calculated molecular mass of WIG (Figure 3A, lane 1). Preincubation of the antibody with an excess of the C-terminal WIG peptide completely abolished recognition of the 53-kD protein (Figure 3A, lane 2).

To test whether the antibody could specifically immunoprecipitate the p53WIG kinase, the alfalfa GSK-3s MsK1 (Pay et al., 1993), MsK4 (C. Jonak and H. Hirt, unpublished results), WIG, and SAMK MAPK (J. Onak et al., 1996) were produced by using in vitro transcription and translation (Figure 3B, lanes 1 to 4, respectively). As depicted in Figure 3B, the WIG antibody immunoprecipitated the p53WIG kinase exclusively (Figure 3B, lane 7); it did not immunoprecipitate the other in vitro-translated alfalfa protein kinases. Thus, the WIG antibody specifically recognizes and immunoprecipitates the p53WIG kinase.

**Rapid and Transient Activation of p53WIG Kinase by Wounding**

The wound-induced expression of the WIG gene suggested to us that WIG may be involved in wound signaling. To obtain more direct evidence for a role of the WIG kinase in wounding, we immunoprecipitated protein extracts of leaves that had been harvested at different times after wounding, using the WIG-specific antibody, and then assayed them for p53WIG kinase activity. Intact leaves contained little active p53WIG kinase (Figure 4, WIG activity, at 0 min), but p53WIG kinase was strongly activated at 5 min after injury. The p53WIG kinase remained fully active until 20 min after wounding. Thereafter, kinase activity slowly returned to basal levels. These results demonstrate that p53WIG kinase is transiently activated by mechanical injury.

When aliquots of the same extracts were used for immunoblotting with the WIG-specific antibody, a single band of 53 kD was detected (Figure 4, WIG protein). In contrast to the wound-induced changes in p53WIG kinase activity and the increase in WIG transcripts, p53WIG protein amounts did not change over the 60-min experimental period.

**WIG Kinase Activation Is Refractory to Restimulation**

To investigate further the transient nature of the wound activation of the WIG kinase, we analyzed whether a second wound stimulus would affect the kinase pathway differently than the first injury did. For this, we applied a second injury at different times after the primary wounding and analyzed the p53WIG kinase activity 5 min later (Figure 5). These assays revealed that rewounding leaves over the first 20 min after the initial injury did not provoke additional activation of p53WIG kinase above that induced by the first stimulus. After 25 min, when p53WIG kinase activity had declined to basal levels, the pathway could not be restimulated at all. By 35 min after the first wounding, however, rewounding was increasingly able to activate the p53WIG kinase pathway again. Thus, wound activation of the WIG pathway is followed by a
Figure 1. Alignment of the Predicted Protein Sequence of Alfalfa WIG, MsK3, Arabidopsis ASKθ, and Rat GSK-3β Protein.

Identical amino acid residues are indicated by black boxes; similar amino acids are indicated with gray boxes. The tyrosine residue important for GSK-3 activity in animals and yeast is conserved in WIG and is marked with an asterisk.
Molecular Regulation of p53<sub>WIG</sub> Kinase Activity

In mammals, regulation of GSK-3 activity is brought about by post-translational mechanisms that involve both protein kinases and phosphatases. As indicated by the constant protein amounts of p53<sub>WIG</sub> kinase before and after wounding (Figure 4), activation of the WIG kinase in leaves is probably mediated by a post-translational mechanism.

To investigate whether wound-induced activation of p53<sub>WIG</sub> depends on transcription, we detached alfalfa leaves at the petioles and, before wounding, preincubated them in Murashige and Skoog (MS) medium containing the transcription inhibitor a-amanitin. To quantify the effect of cycloheximide on protein synthesis, we pulse-labeled leaf pieces with <sup>35</sup>S-methionine after they were incubated with 0, 1, 10, or 100 μM cycloheximide for 2 hr. The degree of inhibition of protein synthesis was determined by scintillation counting of trichloroacetic acid–precipitated total protein or by autoradiography of proteins that had been separated by SDS-PAGE. A linear relationship between the amount of cycloheximide and the amount of incorporated label was obtained: 100 μM cycloheximide inhibited protein synthesis by >95%. Pretreatment of detached leaves with 100 μM cycloheximide for 2 hr before wounding did not affect wound-induced activation of p53<sub>WIG</sub> kinase, but it did block inactivation (Figures 6A and 6B). For a control, we analyzed whether cycloheximide influenced p53<sub>WIG</sub> kinase in the absence of wounding. As shown in Figure 6A, incubation of leaves with 100 μM cycloheximide did not impair wound-induced activation of p53<sub>WIG</sub> kinase but instead resulted in a sustained activation (Figures 6A and 6B). For a control, we analyzed whether a-amanitin affected p53<sub>WIG</sub> kinase in the absence of wounding. As shown in Figure 6A, incubation of unwounded leaves with 100 μM a-amanitin did not induce activation of p53<sub>WIG</sub> kinase. These data indicate that wound-induced activation of p53<sub>WIG</sub> kinase occurs by a post-transcriptional mechanism, whereas inactivation requires gene expression.

To study whether wound-induced activation of p53<sub>WIG</sub> depends on translation, we detached alfalfa leaves at the petioles and, before wounding, preincubated them in MS medium containing the translation inhibitor cycloheximide. Pretreatment with 100 μM cycloheximide before wounding did not impair wound-induced activation of p53<sub>WIG</sub> kinase but instead resulted in a sustained activation (Figures 6A and 6B). For a control, we analyzed whether cycloheximide affected p53<sub>WIG</sub> kinase in the absence of wounding. As shown in Figure 6A, incubation of leaves with 100 μM cycloheximide did not impair wound-induced activation of p53<sub>WIG</sub> kinase but instead resulted in a sustained activation (Figures 6A and 6B).
In this process, de novo synthesis of one or more protein factors is involved. The results obtained by wounding in the absence and presence of α-amanitin and cycloheximide were quantified by Phospholmager analysis. As shown in Figure 6B, the p53WIG kinase was activated by wounding to a similar extent, irrespective of whether transcription or translation was inhibited. On the contrary, transcription and translation were necessary for inactivation of the wound-induced p53WIG kinase, strongly suggesting that de novo synthesis of one or more protein factors is involved in this process.

Inhibition of Translation in Wounded Leaves Induces Sustained Transcript Accumulation

Activation of p53WIG kinase is a rapid, immediate event after wounding and consistently preceded the onset of WIG gene expression. To further study the mechanism of the transcriptional regulation of the WIG gene, we performed RNA gel blot analysis on detached leaves that had been incubated in medium containing cycloheximide before they were cut. As depicted in Figure 7A, inhibition of protein synthesis did not affect the wound-induced onset of WIG gene expression by wounding but resulted in increased accumulation of WIG mRNA. To exclude the possibility that cycloheximide itself could trigger WIG gene expression, we also performed RNA gel blot analysis on detached leaves that had been incubated with cycloheximide alone. As shown in Figure 7B, cycloheximide did not induce expression of the WIG gene. These results reveal that although induction of WIG gene expression is a post-translational process, de novo synthesis of one or more proteins is required to switch off ongoing expression of the WIG gene.

DISCUSSION

Wounding is one of the most severe environmental stresses to which plants can be subjected and may be caused by mechanical injury, pathogen, or herbivore attack. Plants react to wounding by inducing defense responses characterized by the expression of a particular set of genes aimed primarily at preventing invasion by pathogens (Bowles, 1997; Ryan and Pearce, 1998; Baldwin and Preston, 1999). The wound response includes reinforcement of cell walls by deposition of callose, lignin, and hydroxyproline-rich proteins. Moreover, phytoalexins, antimicrobial compounds, protease inhibitors, and lytic enzymes are produced (Bowles, 1990). Most defense responses rely on induction of gene expression that occurs in a complex temporal pattern around the wound site and systemically throughout the whole plant. Because GSK-3s play a prominent role in gene regulation in animals (Plyte et al., 1992; Eastman and Grosschedl, 1999), we investigated whether any of the known alfalfa GSK-3 genes were involved in responses to wounding. We found that the WIG gene, a member of the alfalfa GSK-3 gene family, was rapidly induced in response to mechanical injury. Production of a WIG-specific antibody allowed analysis of the wound-induced processes at the protein and kinase levels. Despite the dramatic accumulation of WIG transcripts after wounding, WIG protein amounts remained constant, suggesting that the protein amounts are tightly regulated by complex intracellular mechanisms. Adding a further level of complexity, WIG showed major changes in kinase activity in wounded leaves, and inhibitor studies revealed that activation of the WIG kinase is mediated by a post-translational mechanism.

All GSK-3s contain a totally conserved threonine residue between subdomains VII and VIII (Figure 1) that is essential for kinase activity (Hughes et al., 1993). Recently, a threonine protein kinase has been isolated from Dictyostelium that can phosphorylate and activate GSK-3 (Kim et al., 1999). By analogy, a threonine kinase could be part of the post-translational activation machinery of plant GSK-3s. Attempts to test this hypothesis with kinase assays of phosphothreonylated antibodies-immunoprecipitated complexes provided inconclusive results because this antibody also binds to the

Figure 4. p53WIG Kinase Is Transiently Activated by Wounding, Whereas Protein Content Stays Constant.

Alfalfa leaves were collected at the times indicated after wounding. (Top) Leaf extracts were immunoprecipitated with the WIG antibody. Activity of the immunoprecipitated p53WIG kinase was determined by in vitro kinase assays with 32P-ATP and myelin basic protein (MBP) as substrate. The phosphorylation of MBP was analyzed by autoradiography after SDS-PAGE. (Bottom) The same protein extracts were used for protein gel blot analysis with the WIG antibody.

Figure 5. Transient Insensitivit of p53WIG to Rewounding.

Leaves were wounded and incubated for the times indicated before a second cutting and incubation for an additional 5 min. p53WIG kinase was immunoprecipitated from 100 μg of leaf protein extracts and assayed for kinase activity with 32P-ATP and MBP as substrate.
phosphotyrosine residues of SAMK, a wound-activated MAPK that shows almost identical kinetics in wounded plants (data not shown). Perhaps the currently produced WIG- and SAMK-specific phosphopeptide antibodies will resolve this question.

Another interesting result obtained in this study concerns the inactivation mechanism of the WIG kinase. After post-translational wound activation of WIG, the kinase stays active for only ~15 to 20 min; thereafter, the kinase becomes inactivated. Because the amounts of WIG protein remain constant, inactivation must be exerted directly on WIG by a post-translational mechanism. Inhibitor studies indicated that the responsible factor for the inactivation of WIG must be a protein that is produced de novo after wounding. This factor could principally be a tyrosine phosphatase, but because mammalian GSK-3s are inactivated through phosphorylation (Sutherland et al., 1993; Stambolic and Woodgett, 1994; Cross et al., 1995), the putative negative regulator of WIG might also be a protein kinase.

Recently, MAPK pathways have been shown to be involved in wound signaling (Seo et al., 1995, 1999; Bögre et al., 1997; Stratmann and Ryan, 1997; Zhang and Klessig, 1998a, 1998b). Comparing the wound response of WIG with that of the SAMK MAPK reveals many similarities. Expression of both the WIG and SAMK genes is induced by wounding. The WIG and SAM kinases both become activated by wounding and show similar transient kinetics (Bögre et al., 1997). Could both kinases be part of the same signal transduction pathway? In some mammalian systems, GSK-3s are part of a MAPK pathway (Eldar-Finkelman et al., 1995). Under these conditions, the MAPK is an upstream negative regulator, regulating GSK-3 indirectly by way of p90rsk kinase (Stambolic and Woodgett, 1994). On the other hand, p90rsk kinase targets a serine in the motif that is absent in all plant GSK-3s, and no plant homolog of p90rsk

**Figure 6.** Inactivation of Wound-Induced p53WIG Activity Requires Transcription and Translation.

(A) Wounded leaves were preincubated for 2 hr in medium containing no inhibitor (w), the transcription inhibitor a-amanitin at 100 μM (α-A + w), or the translation inhibitor cycloheximide at 100 μM (CHX + w). At the times indicated, leaves were collected and used to determine p53WIG activity by immunokinase assays. As a control, detached leaves were also treated with 100 μM cycloheximide (CHX) or 100 μM a-amanitin (α-A) alone.

(B) PhosphorImager analysis of the p53WIG immunokinase assays after wounding in the absence (black columns) or presence (stippled columns) of 100 μM α-amanitin or 100 μM cycloheximide (striped columns). Qualitatively similar results were obtained in three independent experiments.

**Figure 7.** Inhibition of Protein Synthesis Provokes Sustained Wound-Induced Transcription of the WIG Gene.

(A) Detached leaves were preincubated in medium containing 100 μM cycloheximide and subsequently cut with a razor blade.

(B) As a control, detached leaves were treated with 100 μM cycloheximide alone.

At the indicated times, leaves were collected to isolate poly(A)+ RNA. RNA gel blot analysis, using 1 μg of poly(A)+ RNA per lane, was performed simultaneously with probes specific for WIG and Msc27.
kinase was found in a recent two-hybrid screen with WIG (C. Jonak and H. Hirt, unpublished data). Therefore, it remains unclear whether the WIG and SAM kinases lie on the same or on separate pathways.

Wounding plant tissues results in production of jasmonates, which are important mediators for wound-induced gene expression (Bowles, 1997; Creelman and Mullet, 1997; Wasternack et al., 1998). Genetic analysis of WIPK, the wound-induced SAMK homolog in tobacco, revealed that the MAPK is an upstream regulator of the jasmonate synthesis (See et al., 1995, 1999). Consistent with a similar role in alfalfa, jasmonate is unable to activate SAMK (Bögre et al., 1997). We treated leaves with methyl jasmonate to test whether WIG may be a jasmonate-inducible kinase, but no activation of WIG kinase was observed (data not shown). Although these results might indicate that WIG is upstream of the jasmonate pathway, it is equally possible that WIG functions on a separate pathway.

Taken together, our data suggest that WIG likely plays a role in wound signaling, a typical stress response. Most animal GSK-3s have been shown to function in various pathways involved in development, differentiation, and responses to extracellular signals, such as growth factors and insulin. However, the presence of many more GSK-3s in plants suggests that different GSK-3s may perform very different functions; their analysis promises to provide insights into widely different processes, including those found in the animal kingdom.

METHODS

Isolation, Sequence Analysis, and Cloning of WIG

Primers corresponding to two highly conserved domains of MsK1, MsK2, and MsK3 were used to isolate new members of the glycogen synthase kinase 3 (GSK-3) family from alfalfa by polymerase chain reaction (PCR, Pay et al., 1993). The PCR fragment obtained was cloned into pBluescript SK+ (Stratagene). Sequence analysis (T7 Sequencing Kit; Pharmacia) revealed a new type of alfalfa GSK-3, denoted as WIG, which subsequently was used to screen an alfalfa cDNA library prepared from somatic embryos. Two independent clones were isolated. The longer clone was fully sequenced and found to contain an open reading frame of 468 amino acids. The nucleotide and predicted protein sequences are available in the GSDB, DDBJ, EMBL, and NCBI databases under the accession number X82270.

Plant Culture Conditions and Treatments

Alfalfa (Medicago sativa cv Europa) plants were grown in soil in a climate chamber under long-day conditions at 18°C. Leaves were wounded mechanically by cutting the lamina with a razor blade and shock-frozen in liquid nitrogen at the indicated times.

Inhibition of transcription and translation was performed as described (Bögre et al., 1997). To test transcriptional inhibition by α-amanitin, we detached alfalfa leaves at the petioles and incubated them for 2 hr in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 0, 1, 10, or 100 μM α-amanitin (Sigma). RNA gel blot analysis was performed on untreated and α-amanitin-treated samples containing 20 μg of total RNA. After blotting the RNA to nylon membranes, we hybridized the filters with a radiolabeled fragment of the constitutively expressed Msc27 gene (Pay et al., 1992). Imaging with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) was used to quantify the degree of transcription inhibition by α-amanitin. To investigate whether wound-induced activation of p53WIG depended on transcription, we preincubated detached alfalfa leaves for 2 hr in MS medium containing 100 μM α-amanitin.

For translation studies, we detached alfalfa leaves at the petioles and incubated for 2 hr in MS medium containing 0, 1, 10, or 100 μM cycloheximide (Sigma). To quantify the effect of cycloheximide on protein synthesis, we pulse-labeled leaf pieces for 1 hr with 100 μCi 35S-methionine before scintillation counting of trichloroacetic acid-precipitated total protein. For qualitative analysis, we separated proteins using SDS-PAGE and inspected them using autoradiography. To study whether activation of p53WIG by wounding depended on translation, we preincubated detached alfalfa leaves for 2 hr in MS medium containing 100 μM cycloheximide before wounding.

To analyze whether α-amanitin or cycloheximide could activate p53WIG kinase in the absence of wounding, we detached alfalfa leaves at the petioles and preincubated them for 2 hr in MS medium. After the addition of 100 μM α-amanitin or cycloheximide, we determined the activity of p53WIG kinase by immunokinase assays as described below.

RNA Gel Blot Analysis

Poly(A)+ RNA was isolated directly using Dynabeads (Dynal, Great Neck, NY) according to the manufacturer’s protocol. One microgram of poly(A)+ RNA per lane was separated on a denaturing formaldehyde gel. After blotting to nylon membranes, we sequentially hybridized filters with radiolabeled fragments containing the 3′ nontranslated regions of WIG, MsK3 (Pay et al., 1993), and SAMK (previously denoted as MMK4; Jonak et al., 1996). As a control, the blot was hybridized with the coding region of the constitutively expressed Msc27 gene (Pay et al., 1992).

Antibody Production and Specificity Tests

The synthetic peptide VNAPEDLRQA, corresponding to the C terminus of the WIG cDNA, was synthesized and conjugated to a purified derivative protein of tuberculin. Polyclonal antiserum was raised in rabbits. Later analysis of this and other WIG clones indicated that a sequencing mistake had occurred in the original cDNA clone and that the correct corresponding peptide sequence of WIG is VNAPEDLRQR.

For competition assays, we preincubated WIG antibody for 4 hr at 4°C with excess WIG peptide before it was added to the filters. Immunoprecipitation of in vitro-translated protein kinases (T3/T7 coupled in vitro translation kit; Amersham) was performed as described for immunokinase assays.

Preparation of Cell Extracts

Protein extracts were prepared in extraction buffer containing 25 mM Tris-HCl, pH 7.8, 10 mM MgCl2, 15 mM EGTA, 75 mM NaCl, 0.1%
TWEEN 20, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 15 mM β-glycerophosphate, 15 mM 4-nitrophenyl phosphate bis(Tris(hydroxy methyl)aminomethane), and 0.5 mM NaVO₄. After centrifugation at 20,000g for 1 hr, the clear supernatant obtained was used as a total protein source. Protein concentration was determined by the Bio-Rad protein assay system.

**Immunokinase Assays**

Leaf extracts containing 100 μg of total protein were immunoprecipitated with WIG-specific antibody for 6 hr at 4°C. After addition of 30 μL of 50% Protein A-Sepharose beads, samples were incubated for 6 hr. The Sepharose beads were washed three times with wash buffer I (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, and 1% Triton X-100), once with wash buffer II (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 M NaCl, and 1% Triton X-100), and once with kinase buffer (20 mM Hepes, pH 7.4, 15 mM MgCl₂, 5 mM EGTA, and 1 mM DTT). Kinase reactions of the immunoprecipitated kinases were performed in 15 μL of kinase buffer containing 1.5 μg of myelin basic protein (MBP), 10 μM ATP, and 2 μCi of γ-32P-ATP at room temperature for 30 min. Reactions were stopped by addition of SDS sample buffer. Phosphorylation of MBP was analyzed by autoradiography after SDS-PAGE.

**Immunoblotting**

Proteins (40 μg per lane) were separated on 10% SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore). Filters were blocked overnight at 4°C in a solution of 5% nonfat powdered milk before incubation with the secondary antibody for 1 hr at room temperature. After extensive washing of the membranes in TBS-T, the immune reaction products were visualized by enhanced chemiluminescence.

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