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Slow and Prolonged Activation of the p47 Protein Kinase during Hypersensitive Cell Death in a Culture of Tobacco Cells

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To investigate the involvement of protein kinases in the signaling cascade that leads to hypersensitive cell death, we used a previously established system in which a fungal elicitor, xylanase from *Trichoderma viride* (TvX), induces a hypersensitive reaction in tobacco (*Nicotiana tabacum*) cells in culture (line XD6S). The elicitor induced the slow and prolonged activation of a p47 protein kinase, which has the characteristics of a family member of the mitogen-activated protein kinases. An inhibitor of protein kinases, staurosporine, and a blocker of Ca channels, Gd^{3+} ions, both of which blocked the TvX-induced hypersensitive cell death, inhibited the TvX-induced activation of p47 protein kinase. Moreover, an inhibitor of serine/threonine protein phosphatase alone induced both rapid cell death and the persistent activation of the p47 protein kinase. Thus, the p47 protein kinase might be a component of the signal transduction pathway that leads to hypersensitive cell death, and the regulation of the duration of activation of the p47 protein kinase might be important in determining the destiny of tobacco cells.

The active resistance of plants to invasion by pathogens, such as fungi, bacteria, and viruses, is often apparent as the so-called HR of challenged plant cells (Kombrink and Somssich, 1995; He, 1996). The HR can be recognized as the rapid and localized death of cells in response to an avirulent pathogen. It has been observed during most interactions that involve race-specific resistance and in many examples of nonhost resistance (Dangl et al., 1996; He, 1996). Induction of cell death after recognition of an invading pathogen results in the formation of a zone of dead cells around the site of infection.

Several lines of evidence suggest that hypersensitive cell death results from the activation of an intrinsic cell-death program that is encoded within the plant genome (Dangl et al., 1996). Studies have shown that the hypersensitive cell death that occurs in response to certain elicitors requires active plant metabolism and depends on the activity of the host's transcriptional and translational machinery (Yang et al., 1993; He et al., 1994). Hypersensitive cell death is considered to result from the activation of a signal transduction pathway and can be referred to as a type of pcd (Greenberg et al., 1994; Greenberg, 1996; Jones and Dangl, 1996). The term "pcd" refers to a form of cell death that is a normal part of the life cycle of a multicellular organism (Martin JS et al., 1994). In animals pcd is activated during the course of several developmental processes and in response to certain pathogens and environmental stimuli (Haecker and Vaux, 1994). It is controlled by well-characterized gene products, which include activators and inhibitors of pcd (Vaux and Strasser, 1996). Hypersensitive cell death has some of the morphological and biochemical features of apoptosis, which is one of the most widely studied forms of pcd in animals (Mittler and Lam, 1995; Mittler et al., 1995; Levine et al., 1996; Reyerson and Heath, 1996). However, no functional homologs of the molecules that regulate pcd in animals have been identified in plants and it is not clear whether HR-associated pcd is mediated by a mechanism similar to that responsible for some cases of pcd in animal cells.

It is becoming increasingly clear that activation of the SAPK, which is also known as JNK, and of p38 MAP kinase pathways can induce apoptosis in mammalian cells (Canman and Kastan, 1996; Cosulich and Clarke, 1996). For example, an ASK1 (apoptosis signal-regulating kinase 1), a kinase that activates SAPK and p38 MAP kinase pathways and that functions in response to TNF-α is sufficient to induce apoptosis and is required for TNF-induced cell death (Ichijo et al., 1997). Similarly, mitogen-activated/extracellular response kinase kinase kinase, the kinase upstream of the classical JNK cascade, induces apoptosis when expressed ectopically (Johnson et al., 1996). Dominant-negative constituents of the JNK pathway can block stress-induced and TNF-induced apoptosis in several cell lines, observations that suggest that activation of the SAPK pathway might be required for apoptosis in response to these inducers (Verheij et al., 1996).

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Abbreviations: ERK, extracellular signal-regulated kinase; HR, hypersensitive reaction; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; pcd, programmed cell death; PiE, elicitor from *Phytophthora infestans* cell walls; SAPK, stress-activated protein kinase; TNF, tumor necrosis factor; TvX, xylanase from *Trichoderma viride*.

Gene-for-gene complementarity in plant-pathogen interactions implies that an incompatible interaction between a host and a specific pathogen will occur only if the presence of an *avr* (avirulence) gene in the pathogen is matched by the presence of the corresponding *R* (resistance) gene in the host plant. *R* gene-dependent defense mechanisms are often associated with HR or local cell death at sites of entry of pathogens. This genetic interaction between plant and pathogen has led to the current hypothesis that several *R* genes encode protein kinases (Bent, 1996; Suzuki and Shinshi, 1996; Jones, 1997). In addition, it has been reported that HR, induced by an incompatible pathogen or an elicitor of HR, can be prevented by inhibitors of protein kinases (Levine et al., 1994; Zhou et al., 1995). Thus, it seems that a protein kinase cascade might be involved in the intracellular signal transduction that leads to hypersensitive cell death (Bent, 1996; Suzuki and Shinshi, 1996; Jones, 1997).

It has been difficult to dissect the molecular processes of hypersensitive cell death in plants because of the complexity of the intricate plant-pathogen interactions. We recently established a simplified experimental system with which we are able to examine the molecular mechanisms of elicitor-inducible hypersensitive cell death. In our system we used a proteinaceous fungal elicitor that has been shown to elicit the HR in tobacco (*Nicotiana tabacum*) leaves in a cultivar-specific manner (Sharon et al., 1992) and cultured tobacco cells, which can be challenged synchronously with the single molecular inducer of HR (Yano et al., 1998). In this system, the elicitor rapidly induces shrinkage of the cytoplasm, condensation of the nucleus, and hypersensitive cell death, which are accompanied by defense responses typical of HR, such as an oxidative burst and expression of defense genes (Yano et al., 1998). Our results suggest that the elicitor-inducible HR-like response of the cultured tobacco cells is an active process. It appears to be mediated by the activation of a specific signal transduction cascade, which activates the program leading to cell death.

It has been postulated that signals due to elicitors are transduced via a protein kinase cascade in plant cells (Suzuki and Shinshi, 1996). We have shown that protein phosphorylation might be necessary for expression of defense genes (Suzuki et al., 1995). An elicitor-responsive p47 protein kinase has been identified (Suzuki and Shinshi, 1995) and postulated to be a member of the MAP kinase family (Chasan, 1995; Suzuki and Shinshi, 1995; Hirt, 1997; Mizoguchi et al., 1997). This kinase is a convincing candidate for an elicitor-signaling molecule (Suzuki and Shinshi, 1995). The activity of the kinase is barely detectable in untreated cells, but the kinase is activated rapidly, transiently, and strongly (prior to defense responses) upon treatment of cells with the elicitor. In the present study we found that hypersensitive cell death was completely blocked in the presence of a protein kinase inhibitor and that the slow and prolonged activation of p47 protein kinase was induced before the hypersensitive cell death. Because the prolonged activation of p47 protein kinase was well correlated with cell death, it seems possible that the p47 protein kinase might be a component of the signal transduction pathway that leads to hypersensitive cell death.

MATERIALS AND METHODS

Cell Culture and Treatment of Cells with Elicitors and Inhibitors

The conditions for cell culture and the treatment of cells with fungal elicitors were described previously (Suzuki and Shinshi, 1995; Suzuki et al., 1995; Yano et al., 1998). Suspension cultures of tobacco (*Nicotiana tabacum* [line XD6S]) cells were transferred at weekly intervals to fresh Murashige and Skoog medium (Wako Pure Chemical, Osaka, Japan), pH 5.8, that contained 3% Suc and 5 μM 2,4-D. After culture for 4 d, a suspension of line XD6S cells was treated with an elicitor extracted from the cell walls of *Phytophthora infestans* (Suzuki et al., 1995) or with TvX (Sigma). Mes (pH 5.8; final concentration, 25 mM) was also added to the suspension of cells to stabilize the pH of the culture medium. Staurosporine (Kyowa Medex, Tokyo, Japan), $GdCl_3$, calyculin A, or H_2O_2 (Wako Pure Chemical) was included in the medium, with or without TvX, as indicated in the figure legends.

Analysis of Cell Death

Dead cells were quantified by the method described previously (Yano et al., 1998). Cells were stained for 5 min with a 1% solution of Evans blue (Wako Pure Chemical). The suspension of cells was washed five times with the culture medium to remove excess stain. Dye that had bound to dead cells was solubilized in a solution of 50% methanol/1% SDS for 30 min at 50°C and then quantified by monitoring the A_{600}.

Assay of the Oxidative Burst

In the medium of suspension cultures of tobacco cells, H_2O_2 was quantified in terms of the chemiluminescence due to the ferricyanide-catalyzed oxidation of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione [Sigma]), as described by Yano et al. (1998). After cells had been removed by filtration through a cell strainer (Becton Dickinson), an aliquot of the medium and a solution of luminol in 50 mM potassium phosphate buffer (pH 7.9) were mixed in a tube. The reaction was started by the addition of a solution of $K_3(Fe[CN]_6)$ and 14 mM in H_2O_2. The chemiluminescence, recorded with a luminometer (model BLR-201, Aloka, Tokyo, Japan) was integrated for the 30-s period immediately after the start of the reaction.

In-Gel Kinase Assay

Crude extracts were prepared from elicitor-treated cells and subjected to the in-gel kinase and immunoblotting assays as described by Suzuki and Shinshi (1995). The concentration of protein in the extracts was estimated with a protein assay kit (Bio-Rad) with γ-globulin as the standard.

Aliquots of crude extract were subjected to SDS-PAGE on a 10% polyacrylamide gel that had been polymerized with 0.5 mg mL^{-1} MBP (Sigma). After the sample was electrophoresed, SDS was removed and the proteins in the gel were denatured and then renatured; the gel was then incubated in a solution that contained 25 μM ATP (1.85–3.7 of MBq [γ-^{32}P]ATP [Amersham]). The gel was washed extensively, dried, and subjected to autoradiography. The apparent molecular mass of the protein kinase detected on SDS-polyacrylamide gels was estimated with a Rainbow Marker kit (Amersham).

Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting were performed by the method previously described (Suzuki and Shinshi, 1995). The crude extract of elicited line XD6S cells was incubated with the phosphotyrosine-specific monoclonal antibody (4G10, Upstate Biotechnology, Lake Placid, NY) or the ERK1-specific polyclonal antibody (K23, Santa Cruz Biotechnology, Santa Cruz, CA) in an immunoprecipitation buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 5 μg mL^{-1} leupeptin, 5 μg mL^{-1} antipain, 5 μg mL^{-1} aprotinin, 10 mM β-glycerophosphate, 1% Triton X-100, and 0.5% Nonidet P-40) at 4°C for 1 h. After a 2-h binding of antibodies to Protein G PLUS/Protein A-agarose (Oncogene Science, Uniondale, NY), the immunoprecipitates were washed extensively with the immunoprecipitation buffer. The immunoprecipitates were extracted from the agarose bead-protein complex with the SDS sample buffer and subjected to the in-gel kinase assay.

The crude extracts of tobacco cells were fractionated by SDS-PAGE and the proteins were transferred to a PVDF membrane (Immobilon-P, Millipore). The blot was blocked with 10% BSA and then incubated with a phosphotyrosine-specific monoclonal antibody. After extensive washing, the blot was incubated with horseradish peroxidase-conjugated antibodies against mouse IgG (Amersham) and washed again. The antibody-antigen complexes were visualized using an enhanced chemiluminescence system (ECL kit, Amersham). Each blot was exposed to Kodak X-Omat x-ray film.

RNA Gel-Blot Analysis

The isolation of RNA and the RNA gel-blot analysis were performed as described previously (Suzuki et al., 1995). Total RNA was extracted from the tobacco cells, and aliquots of 5 μg of total RNA were denatured, fractionated by electrophoresis on a formaldehyde-agarose gel, and transferred to a Zeta-Probe nylon membrane (Bio-Rad). The RNA on membranes was allowed to hybridize to ^{32}P-labeled cDNA probes that were specific for mRNAs for class I basic chitinase, class II acidic chitinase, and the β-subunit of ATP synthase. Staining with ethidium bromide (Sigma) allowed the visualization of RNA to confirm equal loading of the RNA samples.

RESULTS

Effects of an Inhibitor of Protein Kinase on the HR of Tobacco Cells

For our analysis of the biochemistry of the signal transduction pathway that led to hypersensitive cell death in plant cells, we used a previously established experimental system in which cultured tobacco cells (line XD6S) underwent hypersensitive cell death upon treatment with a fungal proteinaceous elicitor (Yano et al., 1998). In our system, a TvX induced hypersensitive cell death with accompanying changes in the morphology of the cells and their known defense responses. Cell death was monitored by staining with Evans blue, which revealed that treatment of line XD6S cells with TvX resulted, after 3 h, in a rapid increase in the number of dead cells. The number of dead cells reached a maximum within 24 h (Yano et al., 1998).

To examine whether protein kinase activity might be involved in the transduction of the elicitor signal that leads to hypersensitive cell death, we added a protein kinase inhibitor, staurosporine, to the culture medium just before the addition of TvX; we then incubated the cells for 24 h. As shown in Figure 1A, elicitor-induced hypersensitive cell death was slightly enhanced by 1 μM staurosporine but was completely blocked by 10 μM staurosporine. At both concentrations staurosporine also effectively inhibited the oxidative burst (Fig. 1B) and the accumulation of mRNAs for class I basic chitinase and class II acidic chitinase (Fig. 1C). These results suggest that the activity of certain protein kinases was involved in the TvX signal transduction pathway and that the hypersensitive cell death and the defense responses might be differently regulated.

Slow and Prolonged Activation of the p47 Protein Kinase during Hypersensitive Cell Death

To assess the possible involvement of the p47 protein kinase in the transduction of the elicitor signal that led to hypersensitive cell death, we examined whether the p47 protein kinase was activated by TvX in the tobacco cells by using an in-gel kinase assay with MBP as the protein substrate. As shown in Figure 2A, staining with Evans blue detected hypersensitive cell death in a suspension of tobacco cells treated with the TvX but not in a suspension of cells treated with PiE. The activity of p47 protein kinase was barely detectable in untreated cells, but the enzyme was activated rapidly, transiently, and strongly with phosphorylation of a Tyr residue prior to defense responses upon treatment of cells with PiE (Fig. 2, B and C; Suzuki and Shinshi, 1995). By contrast, treatment of tobacco cells with TvX resulted in the delayed and prolonged activation, as well as Tyr phosphorylation, of p47 protein kinase, as shown in Figure 2, B and C. The TvX-induced activation of p47 protein kinase was sustained for at least 4 h; then the activity decreased to the basal level within a further 2 h (Fig. 2B). However, there were no apparent differences in the magnitude of activation and Tyr phosphorylation of the p47 protein kinase between the cells treated with PiE and those treated with TvX (Fig. 2, B and C).

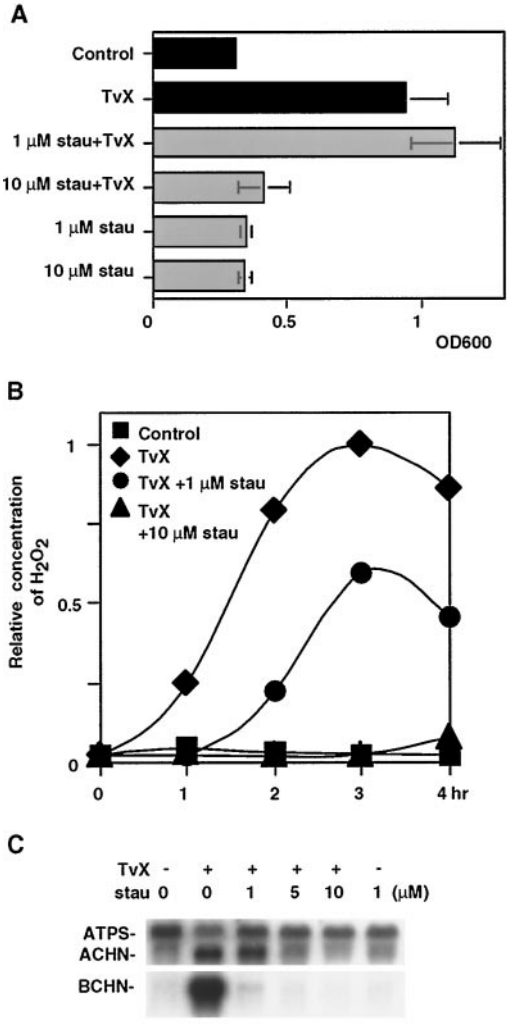

Figure 1. Inhibition of elicitor-inducible cell death and defense responses by a protein kinase inhibitor. Tobacco line XD6S cells were treated with 1 μg mL^{-1} TvX and 1 or 10 μM staurosporine (stau). A, After the sample was incubated for 24 h, cell death was monitored by staining with Evans blue, as described in "Materials and Methods." Data are expressed as the means ± SD of three experiments. B, After incubation for various periods of time, the culture medium was collected and the concentration of H_2O_2 was determined by a chemiluminescence assay, as described in "Materials and Methods." C, After incubation for 5 h, total RNA was prepared and the levels of transcripts of genes for class I basic chitinase (BCHN), class II acidic chitinase (ACHN), and the β-subunit of ATP synthase (ATPS) were analyzed by RNA-blot analysis, as described in "Materials and Methods."

The p47 Protein Kinase Is Recognized by an Antibody Specific to a Mammalian MAP Kinase

Although the molecular identity of the p47 protein kinase remains to be determined, it has been proposed to be a member of the MAP kinase family on the basis of its biochemical features (Chasan, 1995; Suzuki and Shinshi, 1995, 1996; Hirt, 1997; Mizoguchi et al., 1997). Therefore, we examined whether a polyclonal antibody (K23) raised against a mammalian MAP kinase, namely, ERK1, may cross-react with the p47 protein kinase using the immunoprecipitation and the in-gel kinase assay. In the previous report, we showed that a phosphotyrosine-specific monoclonal antibody (4G10) was specifically bound to the active form of the p47 protein kinase in PiE-treated tobacco cells (Suzuki and Shinshi, 1995). As shown in Figure 3, the p47 protein kinase was immunoprecipitated from an extract of PiE- and TvX-treated XD6S cells by K23, as well as by 4G10. This result indicates that the p47 protein kinase is closely related to a mammalian MAP kinase.

Effects of Inhibitors on the Activation of the p47 Protein Kinase by the Elicitor TvX

Our previous results indicated that the activity of an upstream protein kinase(s) is required for the Tyr phos-

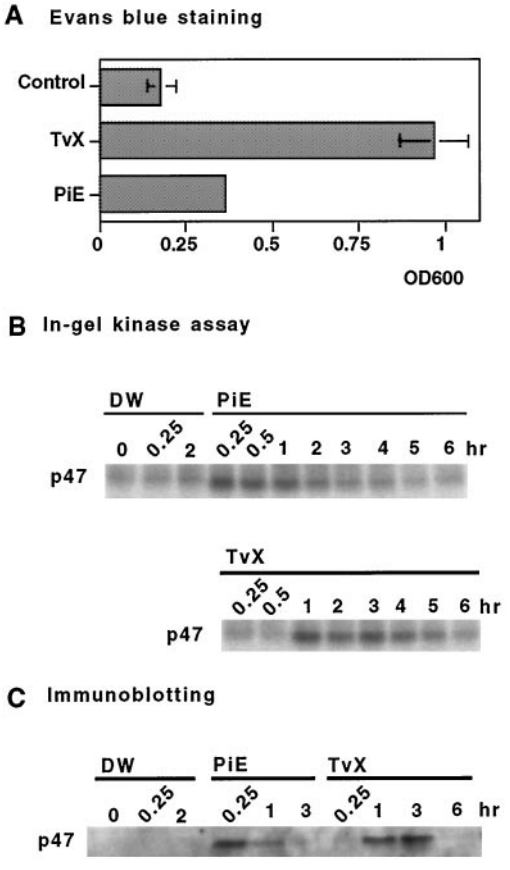

Figure 2. Induction of hypersensitive cell death and activation of the p47 protein kinase by fungal elicitors. Tobacco line XD6S cells were treated with fungal elicitors, namely, a preparation of elicitor from the cell walls of PiE at 50 μg mL^{-1} and TvX at 1 μg mL^{-1}. A, After the sample was incubated for 24 h, cell death was monitored by staining with Evans blue, as described in "Materials and Methods." Data are expressed as the means ± SD of three experiments. B, After incubation for 0 to 6 h, cells were harvested and crude extracts were prepared for the in-gel kinase assay, as described in "Materials and Methods." C, After incubation for 0 to 6 h, cells were harvested and crude extracts were prepared for the immunoblotting with phosphotyrosine-specific antibody, as described in "Materials and Methods." DW, Distilled water.

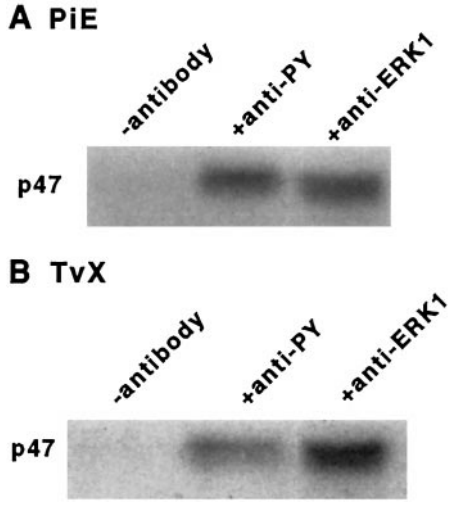

Figure 3. Immunoprecipitation of the p47 protein kinase with the phosphotyrosine-specific antibody (PY) and the ERK1-specific antibody. Tobacco line XD6S cells were treated with fungal elicitors, a preparation of PiE at 50 μg mL^{-1} for 15 min and TvX at 1 μg mL^{-1} for 2 h. After incubation, cells were harvested and crude extracts were prepared for the immunoprecipitation analysis and in-gel kinase assay, as described in "Materials and Methods."

phorylation and activation of the p47 kinase in response to the elicitor (Suzuki and Shinshi, 1995). If a kinase cascade that includes the p47 protein kinase were to participate in elicitor-induced hypersensitive cell death, the TvX-induced activation of p47 protein kinase should also be prevented by staurosporine. Figure 4A shows that staurosporine completely blocked the TvX-induced activation of p47 protein kinase at the same concentration (10 μM) as that at which it inhibited the induction of hypersensitive cell death (Fig. 1A). It is noteworthy that at a lower concentration (1 μM), staurosporine stimulated both hypersensitive cell death and the activation of p47 protein kinase (Figs. 1A and 4A).

It has been suggested that an influx of Ca^{2+} ions across the plasma membrane might be involved in the bacterial induction of hypersensitive cell death in plants (Atkinson et al., 1990; Levine et al., 1996), and such an influx of Ca^{2+} ions has often been implicated in the induction of apoptosis in animal cells (Martin JS et al., 1994). We reported previously that Gd^{3+} ions, which block Ca channels in the plasma membrane, inhibited TvX-induced HR-like responses, including oxidative bursts, the expression of defense genes, and hypersensitive cell death in tobacco cells. Such results suggest that elevation of cytosolic levels of Ca^{2+} ions might play an important role in the signal transduction pathway that leads to hypersensitive cell death (Yano et al., 1998). We also demonstrated that the entry of extracellular Ca^{2+} ions was required for Tyr phosphorylation and activation of the p47 kinase in response to PiE (Suzuki and Shinshi, 1995). Therefore, we examined the effect of Gd^{3+} ions on the TvX-induced activation of p47 protein kinase. As shown in Figure 4B, treatment of line XD6S cells with $GdCl_3$ completely blocked the TvX-induced activation of p47 protein kinase. These results suggest that the TvX-induced activation of p47 protein kinase and hypersensitive cell death both required an influx of Ca^{2+} ions across the plasma membrane.

Induction of Cell Death Is Correlated with the Prolonged Activation of the p47 Protein Kinase

The activation of p47 protein kinase in hypersensitive cell death induced by TvX was apparent as a long-term effect, distinct from the short-term activation by PiE. We reported previously that addition of an inhibitor of protein phosphatases 1 and 2A, calyculin A, to elicitor-treated line XD6S cells induced the sustained activation and the Tyr phosphorylation of the p47 protein kinase (Suzuki and Shinshi, 1995). In the present study we found that treatment of line XD6S cells with calyculin A alone resulted in the prolonged activation of the p47 protein kinase, as shown in Figure 5A. Most cells treated with calyculin A died (Fig. 5B), and the dead cells exhibited the morphological features associated with TvX-induced hypersensitive cell death (data not shown). Calyculin A also induced the oxidative burst (data not shown).

DISCUSSION

Molecular genetic studies, based on gene-for-gene complementarity in plant-pathogen interactions, have revealed that several resistance genes encode protein kinases (Martin GB et al., 1993, 1994; Song et al., 1995; Zhou et al., 1995). In addition, recent biochemical studies demonstrated that the hypersensitive cell death of soybean cells in suspension culture (Levine et al., 1994) and in tobacco leaves (He et al., 1994), induced by incompatible bacteria and an elicitor of the HR, respectively, can be blocked by

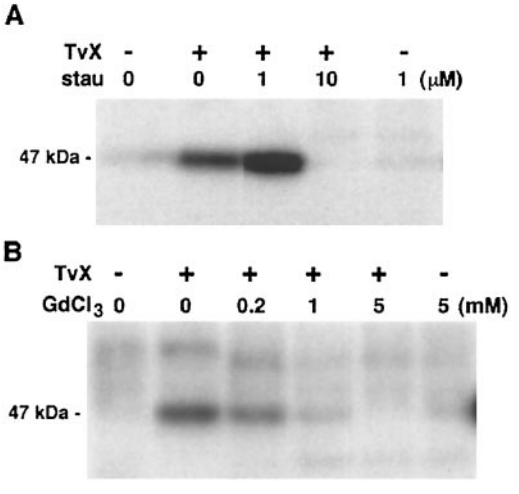

Figure 4. Effects of inhibitors on the elicitor-inducible activation of p47 protein kinase. A, Tobacco XD6S cells were treated with 1 μg mL^{-1} TvX and 1 or 10 μM staurosporine (stau). After incubation for 2 h, cells were harvested and crude extracts were prepared for the in-gel kinase assay, as described in "Materials and Methods." B, Tobacco XD6S cells were treated with 1 μg mL^{-1} TvX and 1 mM $GdCl_3$. After incubation for 2 h, cells were harvested and crude extracts were prepared for the in-gel kinase assay, as described in "Materials and Methods."

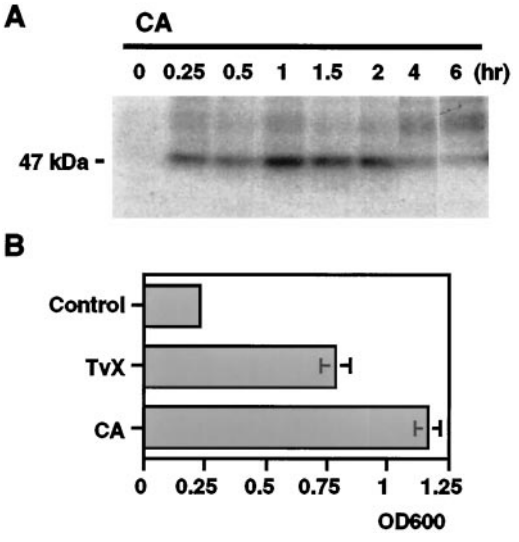

Figure 5. Induction of prolonged activation of p47 protein kinase and cell death by treatment with calyculin A. A, Tobacco line XD6S cells were treated with 1 μM calyculin A (CA). After incubation for various periods of time, cells were harvested and crude extracts were prepared for the in-gel kinase assay, as described in "Materials and Methods." B, Tobacco line XD6S cells were treated with TvX at 1 μg mL^{-1} or with 1 μM calyculin A. After incubation for 24 h, cell death was monitored by staining with Evans blue, as described in "Materials and Methods." Data are expressed as the means ± SD of three experiments.

protein kinase inhibitors. These results suggest that a protein kinase cascade is involved in the recognition of the elicitor signal and in the intracellular signal transduction that leads to hypersensitive cell death (Bent, 1996; Suzuki and Shinshi, 1996; Jones, 1997). However, a correlation of the activity of specific protein kinase to hypersensitive cell death has rarely been demonstrated, although the activation of MAP kinases or MAP kinase-like kinases in response to elicitors or in response to infection by pathogens has been reported (Suzuki and Shinshi, 1995; Ádám et al., 1997; Ligterink et al., 1997; Zhang and Klessig, 1997; Zhang et al., 1998).

In this study we found that the p47 protein kinase, which has the characteristics of MAP kinases, was slowly and extensively activated during hypersensitive cell death induced by an HR elicitor. We showed that a protein kinase inhibitor, staurosporine, inhibited the TvX-induced cell death at 10 μM and that a protein phosphatase inhibitor, calyculin A, induced the rapid cell death at 1 μM. These results suggest that the activity of certain protein kinases in a protein kinase cascade, which is possibly negatively regulated by the activity of protein phosphatase 1 and/or 2A, might be involved in the regulation of TvX-induced hypersensitive cell death. In addition, we found that 10 μM staurosporine blocked the activation of p47 protein kinase and that 1 μM calyculin A induced its activation. Moreover, 1 μM staurosporine enhanced the activation of p47 protein kinase and the cell death induced by TvX. These findings are consistent with the following model: the p47 protein kinase mediates the phosphorylation-dependent signal transduction pathway in response to TvX, which leads to defense responses and hypersensitive cell death, but staurosporine and calyculin A may inhibit multiple protein kinases and protein phosphatases, respectively, in tobacco cells.

We demonstrated previously that the p47 protein kinase was rapidly and transiently activated prior to the induction of defense responses in tobacco cells that had been treated with PiE (Suzuki and Shinshi, 1995). However, transfer of suspension-cultured cells to a new plastic dish induced short-term and limited activation of the p47 protein kinase, although it did not induce any defense responses in tobacco cells (Suzuki and Shinshi, 1995). Therefore, the regulation of the actual duration of activation of p47 protein kinase might be crucial in the determination of subsequent responses of the tobacco cell, such as hypersensitive cell death and defense responses, because the magnitude of activation of the p47 protein kinase was similar for the two fungal elicitors. It is reasonable to postulate that very short-term and limited activation of p47 protein kinase by the (probably mechanical) transfer stress, for example, is insufficient for induction of downstream events in the elicitor-initiated signal transduction cascade. By contrast, the elicitor-induced rapid and transient activation of the p47 protein kinase might be sufficient for initiation of defense responses, such as the oxidative burst and expression of defense genes. Furthermore, it is plausible that the elicitor-induced slow and prolonged activation of the p47 protein kinase might be a prerequisite for hypersensitive cell death.

A similar paradigm has been suggested for the ERK pathway to explain its role in both proliferative and differentiation-related responses (Marshall, 1995). In addition, several studies have revealed the importance of the duration of activation of JNK/SAPK and/or p38 MAP kinase in the determination of cell fate (Xia et al., 1995; Chen et al., 1996a, 1996b; Goillot et al., 1997). For example, in Jurkat T-cells, the T-cell activation signal induced the rapid and transient activation of JNK and the proliferation of T-cells. In contrast, a lethal dose of γ-radiation or UV-C induced the delayed and persistent activation of JNK and apoptotic cell death (Chen et al., 1996a, 1996b). In human B lymphocytes, apoptosis and sustained activation of SAPK and p38 MAP kinase, which were induced by the cross-linking of membrane IgM, were inhibited by a Ca channel blocker (Graves et al., 1996). In this study we have also shown that a Ca channel blocker prevented not only the sustained activation of the p47 protein kinase but also the induction of hypersensitive cell death in XD6S cells treated with TvX. Thus, the p47 protein kinase in tobacco cells appears to be functionally similar to SAPK/JNK and/or p38 MAP kinase in mammalian cells; the p47 protein kinase may play a role as a component of the elicitor signal transduction.

Our previous results suggested that the activity of upstream protein kinases is required for the PiE-induced Tyr phosphorylation and the activation of p47 kinase, that the activation of p47 protein kinase is regulated posttranslationally, and that both synthesis of protein de novo and protein phosphatase activity are required for the attenuation of p47 kinase activity (Suzuki and Shinshi, 1995). The

immediate and transient nature of the activation that occurs in response to fungal elicitors appears to be a common feature of various MBP kinases, including MAP kinases (Suzuki and Shinshi, 1995; Ádám et al., 1997; Ligterink et al., 1997; Zhang et al., 1998). In our system we are interested in the mechanism of induction of the delayed and prolonged activation of the p47 protein kinase by TvX. This process could involve either prolonged activation of upstream kinases or inhibition and/or down-regulation of specific protein phosphatases. The observation that calyculin A induced the prolonged activation of p47 protein kinase, defense responses, and cell death suggests possible posttranslational negative modulation of the activity of a constitutive p47 protein kinase and its upstream kinases via dephosphorylation-mediated down-regulation.

We have demonstrated that 1 μM staurosporine inhibits the PiE-induced activation of the p47 protein kinase (Suzuki and Shinshi, 1995) and the expression of defense genes (Suzuki et al., 1995). In the present study the staurosporine at the higher of the two tested concentrations (10 μM) also prevented the TvX-induced activation of the p47 protein kinase and the defense responses. At the lower concentration (1 μM) of staurosporine, however, TvX-induced cell death and the activation of p47 protein kinase were enhanced but TvX-induced defense responses were inhibited. These results show that the PiE- and TvX-induced defense responses and the TvX-induced hypersensitive cell death might require the activation of p47 protein kinase and its phosphorylation by upstream kinase(s), but the particular elicitors might differentially activate the p47 protein kinase via different pathways. The active p47 protein kinase might also activate other downstream kinases, which have varying sensitivities to staurosporine and which are involved in the induction of varying cellular responses.

The oxidative burst that occurs during plant-microbe interactions has been shown to be induced via a protein kinase cascade. The reactive oxygen species from that oxidative burst have been implicated in the elicitor signal transduction that leads to multiple phenomena, including defense responses and hypersensitive cell death (for reviews, see Doke et al., 1996; Low and Merida, 1996). Therefore, we have investigated the possible involvement of reactive oxygen species in TvX-induced cell death. We have found that TvX induces activation of p47 protein kinase(s) and the induction of chitinase genes, as well as the hypersensitive cell death even in the presence of oxidant inhibitors (such as catalase, diphenylene iodonium, or *N*-acetylcysteine), which completely block the accumulation of H_2O_2 from the oxidative burst (A. Yano, K. Suzuki, and H. Shinshi, unpublished results).

Several cDNAs that encode MAP kinases have been isolated from tobacco (Wilson et al., 1993, 1995; Seo et al., 1995; Zhang and Klessig, 1997). Recently, Zhang et al. (1998) demonstrated that a MAP kinase of 48 kD was activated in response to fungal elicitors in tobacco cells. Further biochemical and molecular analyses after the identification of the p47 protein kinase will help us to elucidate the role of this kinase in the elicitor signal transduction that leads to defense responses and hypersensitive cell death and thereby to clarify any relationships between this kinase and other MAP kinases.

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