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Race-Specific Elicitors of *Cladosporium fulvum* Promote Translocation of Cytosolic Components of NADPH Oxidase to the Plasma Membrane of Tomato Cells

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The effect of race-specific elicitors on NADPH oxidase was examined in vivo by treating tomato cells with elicitor-containing intercellular fluids prepared from infected tomato leaves inoculated with specific *Cladosporium fulvum* races. Treatment of *Cf-4* or *Cf-5* cells with intercellular fluids from incompatible but not from compatible races of *C. fulvum* increased oxidase activity and the amount of p67-*phox*, p47-*phox*, and rac2 in the plasma membrane. Comparison of these three components in the cytosol and plasma membrane indicated that elicitors promoted the translocation of cytosolic components of NADPH oxidase to the plasma membrane of tomato cells carrying the appropriate resistance gene. Protein kinase C activators and inhibitors did not affect enzyme activity or the binding of these three components to the plasma membrane. In contrast, staurosporine, calmodulin antagonists, and EGTA inhibited elicitor-induced oxidase activity and the translocation of the cytosolic components. The assembly process involves a Ca^{2+}-dependent protein kinase that catalyzes the phosphorylation of p67-*phox* and p47-*phox*, facilitating their translocation to the plasma membrane. Our data suggest that although both plants and animals share common elements in eukaryotic signal transduction, the involvement of different protein kinases mediating the activation of phosphorylation of p67-*phox* and p47-*phox* may reflect the unique spatial and temporal distribution of signal transduction pathways in plants.

INTRODUCTION

Plant defenses against pathogen attack involve an array of inducible responses that contribute to resistance in the host plant. The interaction between the leaf mold pathogen *Cladosporium fulvum* and tomato has been extensively investigated (De Wit, 1992). It is now well documented that plant disease resistance involves a resistance (*R*) gene in the plant that responds specifically to the product of a single avirulence (*avr*) gene in the pathogen (Keen, 1990; De Wit, 1992). The recognition of elicitors by putative receptors is proposed to initiate a cascade of events leading to the induction of defense responses in plant cells. Previous work with *Cf*-5 cell suspension cultures showed that changes in the phosphorylation status of the host plasma membrane H^+-ATPase (Vera-Estrella et al., 1994a; Xing et al., 1996), the activity of plasma membrane Ca^{2+} channels (Gelli et al., 1997), and the plasma membrane redox activities (Vera-Estrella et al., 1994b) could be involved in the defense response of tomato cells to *C. fulvum* attack. A heterotrimeric G protein appears to be a key mediator of the signal transduction processes described above (Xing et al., 1997).

Among plant defense responses to pathogen attack, the rapid production of hydrogen peroxide (H_2O_2) and its probable precursor, superoxide (O_2^-), may play an important role. The release of these active oxygen species (AOS), termed the oxidative burst, may affect the attacking pathogen and the host plant cells at the infection site, thereby limiting the spread of the pathogen (Mehdy, 1994). Speculation on the involvement of a plasma membrane–associated NADPH oxidase in the oxidative burst has been published (Apostol et al., 1989; Vera-Estrella et al., 1992; Baker et al., 1993; Legendre et al., 1993b; Lamb, 1994; Mehdy, 1994; Doke and Miura, 1995). Studies of this enzyme in phagocyte cells indicated that an assembly process involving two plasma membrane subunits and three cytosolic components was triggered in these cells upon pathogen attack (Segal and Abo, 1993; Jones, 1994).

The oxidase electron transport chain, located in the plasma membrane, comprises a cytochrome *b* heterodimer (gp91-*phox* and p22-*phox*). It is nonfunctional until at least three proteins, p47-*phox*, p67-*phox*, and a monomeric G protein, rac (and possibly others), move from the cytosol to dock on cytochrome *b*. The docking process involves the interaction of SH_3 domains on p47-*phox* or p67-*phox* with a proline-rich sequence on p22-*phox*. These SH_3 domains may become exposed after phosphorylation of p47-*phox* by protein kinase C (PKC). After the docking process, the electron-transporting component is able to transfer electrons from NADPH to oxygen.

Evidence is emerging to support the similarity of NADPH oxidase in plant cells and phagocytic animal cells. Levine et

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al. (1994) showed the cross-reactivity of antibodies raised against a component of the mammalian NADPH oxidase subunits (p22-*phox*) with proteins of similar molecular mass in soybean membranes. Elicitor induction of the oxidative burst in soybean, parsley, and Arabidopsis was inhibited by diphenylene iodonium, a suicide inhibitor of the mammalian NADPH oxidase (Levine et al., 1994; Nurnberger et al., 1994; Desikan et al., 1996; Dwyer et al., 1996). Antibodies raised against human neutrophil p47-*phox* and p67-*phox* cross-reacted with proteins of the same molecular mass in extracts from soybean, cotton, and Arabidopsis, and plant species that expressed proteins immunologically related to p47-*phox* and p67-*phox* also generated an oxidative burst (Desikan et al., 1996; Dwyer et al., 1996). In mammalian systems, activation of the oxidase is associated with movement of rac to the membranes (Segal and Abo, 1993). There is also evidence for protein kinase involvement, because the oxidative burst is blocked by protein kinase inhibitors and stimulated by an inhibitor of protein phosphatase 2A (Levine et al., 1994; Chandra and Low, 1995; Dwyer et al., 1996). It is currently believed that at least in animal phagocytic cells, PKC is needed to phosphorylate p47-*phox* (Segal and Abo, 1993; Jones, 1994).

The involvement of AOS (Vera-Estrella et al., 1992, 1994b) and a PKC-like protein kinase (Xing et al., 1996) has been demonstrated in the interaction between the leaf mold pathogen *C. fulvum* and tomato cells. This suggested the possibility that NADPH oxidase on the tomato plasma membrane may act in a manner similar to that of phagocytic cells when tomato cells are treated with elicitors isolated from *C. fulvum*. Here, we examine the effect of elicitor preparations isolated from *C. fulvum* on NADPH oxidase activity of the host plasma membrane and the translocation of p47-*phox*, p67-*phox*, and rac in host cells. We also examine the possible role of protein kinases in the translocation of these three enzyme components and the regulation of NADPH oxidase activity.

RESULTS

Effects of Race-Specific Elicitors on NADPH Oxidase Activity and on the Amount of p67-*phox*, p47-*phox*, and rac on Host Plasma Membrane

In the study of plant–pathogen interactions, several enzyme systems have been proposed as the source of AOS production. The best characterized example of active oxygen involvement in disease resistance is the respiratory burst of mammalian phagocytes that is responsible for the killing of foreign bacteria. The respiratory burst is attributed to the activation of a host plasma membrane–bound NADPH oxidase that transfers electrons from NADPH on the interior of the cell to molecular oxygen on the outside, leading to the generation of O_2^- (Segal and Abo, 1993). Speculation is that a plasma membrane–localized NADPH oxidase similar to that in the phagocyte is responsible for the oxidative burst in plants (Vera-Estrella et al., 1992; Baker et al., 1993; Lamb, 1994; Mehdy, 1994; Doke and Miura, 1995).

The signaling pathway in plants closely parallels that established for the mammalian oxidative burst. Similarities include the involvement of G proteins (Legendre et al., 1993a; Vera-Estrella et al., 1994b) and a need for continuous Ca^{2+} influx and protein kinase activity (Schwacke and Hager, 1992; Baker et al., 1993; Doke and Miura, 1995). Recent immunological studies further indicate that plant cells contain molecular components for the assembly of NADPH oxidase, including p67-*phox*, p47-*phox* (Desikan et al., 1996; Dwyer et al., 1996), gp91-*phox*, and p22-*phox* (Levine et al., 1994; Tenhaken at al., 1995). In this study, the effect of elicitors from *C. fulvum* on NADPH oxidase activity of the plasma membrane of tomato cells was examined, with emphasis on the assembly of the enzyme components.

The effect of race-specific elicitors was examined in vivo by treating tomato cells with intercellular fluid (IF) prepared using incompatible or compatible races of *C. fulvum*. After treatment of the cells, the plasma membranes were isolated and NADPH oxidase activity was measured, with several of its components being determined immunologically (Figure 1A). The treatment of *Cf-4* cells with the IFs from race 2.3 and race 5 (both incompatible on plants with the *Cf-4* gene) induced a significant increase in enzyme activity (Figure 1A) and an increase in the amount of the immunologically detected p67-*phox*, p47-*phox*, and rac2 (Figures 1B to 1D, respectively). No changes were observed when *Cf-4* cells were treated with IFs from *Cf-4*–compatible races 4 and 2.4.5.9.11 (Figures 1A to 1D). Race cultivar specificity was also observed in *Cf-5* cells (Figures 1E to 1H). IFs from *Cf-5*–incompatible races 2.3 and 4 caused increased enzyme activity and an increase in the immunologically detectable amount of p67-*phox*, p47-*phox*, and rac2, whereas IFs from compatible races, races 5 and 2.4.5.9.11, caused no change in any of these parameters. Such IF treatment did not change the total cellular amount of p67-*phox*, p47-*phox*, and rac2 (data not shown).

Possible Involvement of Other rac Species

In phagocytic cells, two major types of rac have been found to operate as a component of NADPH oxidase. rac1 and rac2, which share 92% amino acid sequence homology, probably play similar roles in different cells (Abo et al., 1991). Specific antibodies raised against rac1 and rac2 were used to identify rac in the plasma membrane and cytosolic fractions of tomato cells. rac2 could be immunologically detected in plasma membranes from control cells. Treatment of the cells induced a significant increase in the amount of rac2 bound to the membrane (Figure 2B). rac1 was not detected in plasma membrane (Figure 2A) or cytosol of control or treated cells (data not shown).

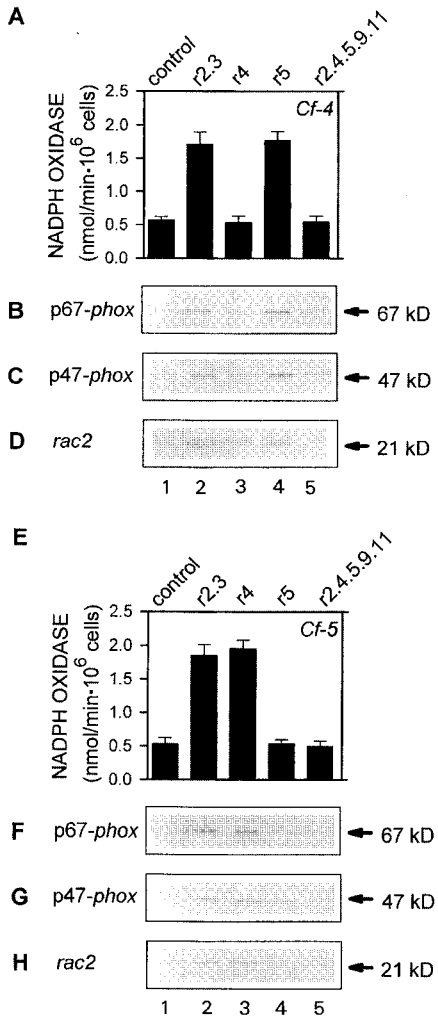

Figure 1. NADPH Oxidase Activity and Immunodetection of p67-*phox*, p47-*phox*, and rac2 after Treatment with Elicitor Preparations (IF) from *C. fulvum*.

(A) to **(D)** *Cf-4* cells were treated for 30 min with H_2O (control), race 2.3 (r2.3), race 4 (r4), race 5 (r5), and race 2.4.5.9.11 (r2.4.5.9.11) IFs (lanes 1 to 5, respectively, in **[B]** to **[D]**). After plasma membrane isolation, the membranes were assayed for NADPH oxidase activity **(A)**, or the membrane proteins were separated by SDS-PAGE and detected by immunoblotting with antibodies raised against p67-*phox* **(B)**, p47-*phox* **(C)**, and rac2 **(D)**.

(E) to **(H)** correspond to **(A)** to **(D)**, respectively, except that *Cf-5* cells were used rather than *Cf-4* cells. Lane settings in **(F)** to **(H)** are as given for **(B)** to **(D)**, respectively.

Numbers at right represent the molecular masses of the protein standards in kilodaltons. Values for NADPH activity are the means ± SE (n = 4). Calculations were based on 10^6 cells equivalent of plasma membrane proteins. Blots are representative of three independent experiments.

Movement of p67-*phox*, p47-*phox*, and rac2 from the Cytosol to the Plasma Membrane When Treated with Fungal Elicitors

Activation of NADPH oxidase in phagocyte cells involves translocation of p67-*phox*, p47-*phox*, and rac from the cytosol to the plasma membrane (Segal and Abo, 1993; Jones, 1994). Their translocation has been used as an indicator of the activation of these proteins in intact cells by upstream signals (Dorseuil et al., 1995). A similar translocation of p67-*phox*, p47-*phox*, and rac2 from the cytosol to the plasma membrane was observed when tomato cells were elicited by fungal elicitors (Figures 3A to 3C). The decrease of immunologically related proteins in the cytosol corresponded to their increase in the plasma membrane.

Association of p67-*phox*, p47-*phox*, and rac2 with Membrane Cytoskeleton

Treatment with Triton X-100 separates membranes into a Triton X-100–insoluble fraction (generally equated with the membrane cytoskeleton) and a Triton X-100–soluble fraction (containing the noncytoskeleton membrane proteins) (Fox, 1985; Quinn et al., 1989). Analysis of cytoskeletal and noncytoskeletal fractions by immunoblotting showed that Triton X-100–soluble fractions, obtained either from control or from treated membranes, contained no detectable p67-*phox*, p47-*phox*, and rac2 but that these components were present in Triton X-100–insoluble fractions (Figures 4A to 4C). This could reflect either a physiological association with the cytoskeleton or an intrinsic insolubility in the cytoskeleton. To distinguish between these possibilities, we treated the plasma membrane with DNase I. Treatment with DNase I, combined with high-speed centrifugation, solubilizes proteins associated with the cytoskeleton by removing actin

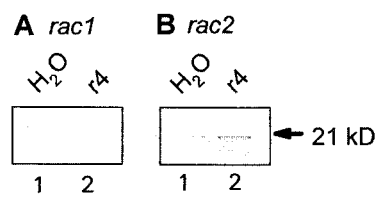

Figure 2. Relative Levels of rac1 and rac2 in the Plasma Membrane after Elicitor Treatment.

(A) *Cf-5* cells were treated for 30 min with H_2O (control) and race 4 (r4) IF (lanes 1 and 2, respectively). After plasma membrane isolation, the samples were separated by SDS-PAGE and detected by immunoblotting with antibodies raised against rac1.

(B) corresponds to **(A)**, except that antibodies raised against rac2 were used for immunoblotting.

The 21 kD in **(B)** represents the molecular mass of the protein standard. Blots are representative of three independent experiments.

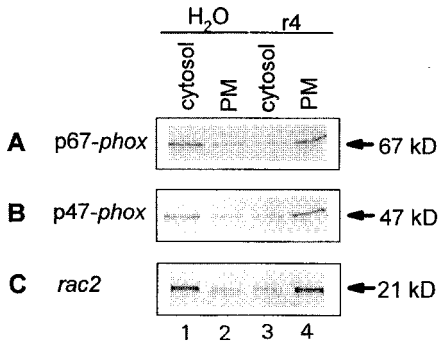

Figure 3. Transfer of p67-*phox*, p47-*phox*, and rac2 from the Cytosol to the Plasma Membrane.

(A) *Cf-5* cells were treated for 30 min with H_2O (control) and race 4 (r4) IF. After plasma membrane and cytosol isolation, the samples were separated by SDS-PAGE and detected by immunoblotting with antibodies raised against p67-*phox*.

(B) corresponds to **(A)**, except that antibodies raised against p47-*phox* were used for immunoblotting.

(C) corresponds to **(A)**, except that antibodies raised against rac2 were used for immunoblotting.

Lanes 1 contain cytosol from control cells; lanes 2, plasma membrane (PM) of control cells; lanes 3, cytosol of race 4 IF–treated cells; lanes 4, PM of race 4 IF–treated cells. Numbers at right represent the molecular masses of the protein standards in kilodaltons. Blots are representative of three independent experiments.

filaments via depolymerization but should not affect proteins that are intrinsically insoluble in the cytoskeleton buffer (Fox, 1985; Carlier, 1991; El Benna et al., 1994). Figures 4A to 4C show that p67-*phox*, p47-*phox*, and rac2 were solubilized by DNase I, indicating that they are associated with the membrane cytoskeleton but are not intrinsically insoluble membrane proteins.

Time Course of the Association of p67-*phox*, p47-*phox*, and rac2 with the Plasma Membrane When Treated with the Elicitor

The increase in the amount of p67-*phox*, p47-*phox*, and rac2 in tomato plasma membrane was monitored during the first 30 min after elicitor treatment. The increase in the amount of membrane-bound proteins could be detected 10 min after treatment of tomato cells with the elicitor and continued for at least 30 min (Figures 5A to 5C).

Effects of Protein Kinase Activators and Inhibitors on Host Plasma Membrane NADPH Oxidase Activity and on the Translocation of p67-*phox*, p47-*phox*, and rac2

The PKC-mediated phosphorylation of p67-*phox* and p47-*phox* has been long suspected in the activation of neutrophil NADPH oxidase (Segal and Abo, 1993; Jones, 1994; Leto et al., 1994). Phorbol 12-myristate 13-acetate (PMA), a PKC activator, has often been used to activate the plasma membrane NADPH oxidase in vivo or in cell-free systems (El Benna et al., 1994; Heyworth et al., 1994; Dorseuil et al., 1995; Leusen et al., 1995). PMA was also shown to generate AOS in Arabidopsis cells (Desikan et al., 1996). In our tomato cells, the effect of PKC activators on NADPH oxidase activity was studied by their addition to cells for 30 min. Neither PMA nor SC-10 activated NADPH oxidase activity in the absence of the elicitor (Figure 6A). The addition of PKC inhibitors (bisindolylmaleimide, calphostin C, and chelerythrine) to cells 15 min before the addition of the elicitor did not inhibit the elicitor-induced increase of NADPH oxidase activity (Figure 6A). In contrast, the enhanced enzyme activity was completely inhibited by staurosporine (a general inhibitor of protein kinases). Immunoblotting indicated that only staurosporine, but not PKC activators or inhibitors, affected the amount of p67-*phox*, p47-*phox*, and rac2 bound to the plasma membrane of the host cells (Figures 6B to 6D, respectively). These observations suggest that a PKC-like protein ki-

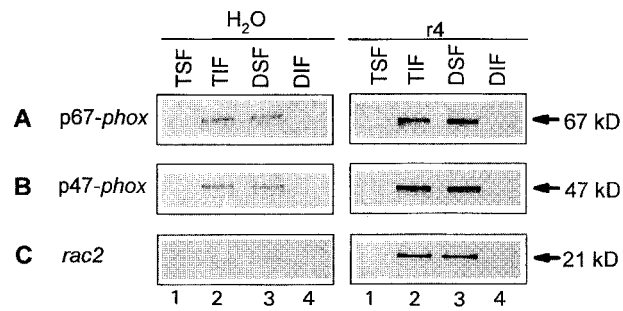

Figure 4. Association of p67-*phox*, p47-*phox*, and rac2 with the Membrane Cytoskeleton.

Cf-5 cells were treated for 30 min with H_2O (control) and race 4 (r4) IF.

(A) Plasma membranes were isolated and incubated for 30 min at 4°C in cytoskeleton buffer. Triton X-100–insoluble and Triton X-100–soluble fractions were separated by centrifugation at 180,000*g* for 2 hr at 4°C. Triton X-100–insoluble fractions were further treated with the DNase I–containing cytoskeleton buffer for 2 hr at 4°C. DNase I–washed fractions were obtained by centrifugation at 180,000*g* for 2 hr at 4°C. Proteins were separated by SDS-PAGE and detected by immunoblotting with antibodies raised against p67-*phox*.

(B) corresponds to **(A)**, except that antibodies raised against p47-*phox* were used for immunoblotting.

(C) corresponds to **(A)**, except that antibodies raised against rac2 were used for immunoblotting.

Lanes 1 contain Triton X-100–soluble fraction (TSF); lanes 2, Triton X-100–insoluble fraction (TIF); lanes 3, DNase I–soluble fraction (DSF); lanes 4, DNase I–insoluble fraction (DIF). Numbers at right represent the molecular masses of the protein standards in kilodaltons. Blots are representative of three independent experiments.

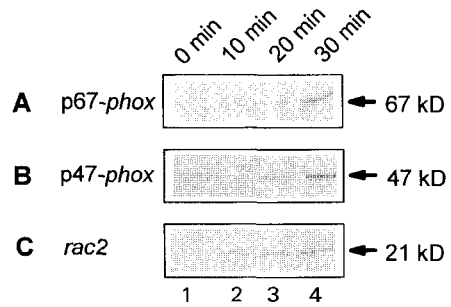

Figure 5. Time Course of the Association of p67-*phox*, p47-*phox*, and rac2 with the Plasma Membrane in Elicitor-Treated Cells.

(A) *Cf-5* cells were treated with race 4 IF for 0, 10, 20, and 30 min (lanes 1 to 4, respectively). After plasma membrane isolation, the samples were separated by SDS-PAGE and detected by immunoblotting with antibodies raised against p67-*phox*.

(B) corresponds to **(A)**, except that antibodies raised against p47-*phox* were used for immunoblotting.

(C) corresponds to **(A)**, except that antibodies raised against rac2 were used for immunoblotting.

Numbers at right represent the molecular masses of protein standards in kilodaltons. Blots are representative of three independent experiments.

nase(s) may not be involved in the assembly of NADPH oxidase during the activation of the enzyme by fungal elicitors.

The effect of calmodulin (CaM) antagonists and EGTA on NADPH oxidase activity was also studied. The addition of CaM antagonists W-7 (Itoh and Hidaka, 1984), trifluoperazine (TFP) dimaleate (Massom et al., 1990), and EGTA to the cell cultures 15 min before the addition of the elicitor significantly inhibited the elicitor-induced increase in NADPH oxidase activity (Figure 6E). W-7, TFP dimaleate, and EGTA also inhibited the elicitor-induced increase of translocated p67-*phox*, p47-*phox*, and rac2 to the plasma membrane (Figures 6F to 6H).

The involvement of a Ca^{2+}-mediated and CaM-mediated protein kinase in the elicitor-induced increase of NADPH oxidase activity was further indicated by the in vivo phosphorylation of cellular proteins. Cells were in vivo labeled with ^{32}P-phosphorus. After labeling, the cells were elicited in the presence of EGTA, W-7, or bisindolylmaleimide, and p47-*phox* and p67-*phox* were immunoprecipitated. The addition of EGTA or W-7 inhibited the elicitor-induced phosphorylation of p47-*phox* and p67-*phox*, whereas bisindolylmaleimide did not affect their phosphorylation (Figures 7A and 7B).

DISCUSSION

As a possible key element in the production of AOS and plant defense against pathogen attack, NADPH oxidase has been studied for more than a decade. It is now generally accepted that after the perception of elicitors by receptors and the transduction by G proteins, the signal is further amplified via increased intracellular Ca^{2+} due to the opening of Ca^{2+} channels, leading to the activation of a protein kinase(s) that activates NADPH oxidase by phosphorylation (Mehdy, 1994). Detailed studies of how NADPH oxidase is activated in plants have been difficult to perform, but now results from phagocytes and neutrophils present a testable mechanism as well as probes to study its regulation.

The tomato–*C. fulvum* interaction presents an ideal model to study the activation of NADPH oxidase because (1) an oxidative burst is an early indicator of an incompatible (resistant) response; (2) elicitors that are direct products of the *avr* genes can reproduce the defense response and the oxidative burst; and (3) elicitor-induced effects on isolated plasma membranes have been reported (Vera-Estrella et al., 1994a, 1994b; Xing et al., 1996, 1997; Gelli et al., 1997). After treatment of tomato cells with IFs from *C. fulvum*, NADPH oxidase activity in the isolated plasma membranes was determined, and the levels of the three proposed cytosolic proteins on plasma membranes were immunologically detected. With the combination of two different tomato cell lines (*Cf-4* and *Cf-5*) and IF preparations isolated from four different *C. fulvum* races, only the "incompatible" combinations showed increased NADPH oxidase activity. In addition, the quantitative changes of p67-*phox*, p47-*phox*, and rac2 in the plasma membranes were correlated with enzyme activity. Two major rac proteins, rac1 and rac2, are known to participate in the activation of NADPH oxidase in phagocytic cells (Abo et al., 1991; Knaus et al., 1991). When membranes were tested with antibodies raised against rac1 and rac2, only rac2 was detectable, suggesting that rac2 but not rac1 was responsible for the activation of NADPH oxidase in tomato cells.

The increase of the three proposed cytosolic proteins on the plasma membranes was detectable 10 min after elicitor treatment and continued for at least 30 min. This increase was shown to be the result of their movement from the cytosol to the plasma membrane. By treatment of the plasma membranes with Triton X-100 or DNase I, these three proteins were shown to be associated with the cytoskeleton but not to be intrinsically insoluble membrane proteins. In neutrophils, it is clear that the membrane skeleton plays a role in NADPH oxidase assembly and/or regulation (Jesaitis et al., 1986; Quinn et al., 1989; Nauseef et al., 1991). Quinn et al. (1989) found that NADPH oxidase activity was associated with the Triton X-100–insoluble cytoskeleton of human neutrophils. This activity was confined to a minor subfraction of the plasma membrane in activated neutrophils, and this "heavy subfraction" was enriched in actin and fodrin (Quinn et al., 1989). In addition, the specific components of NADPH oxidase were confirmed to associate with the Triton X-100–insoluble cytoskeleton of activated neutrophils (Quinn et al., 1989; Nauseef et al., 1991; El Benna et al., 1994). The elicitor-induced association of p47-*phox*, p67-*phox*, and rac2

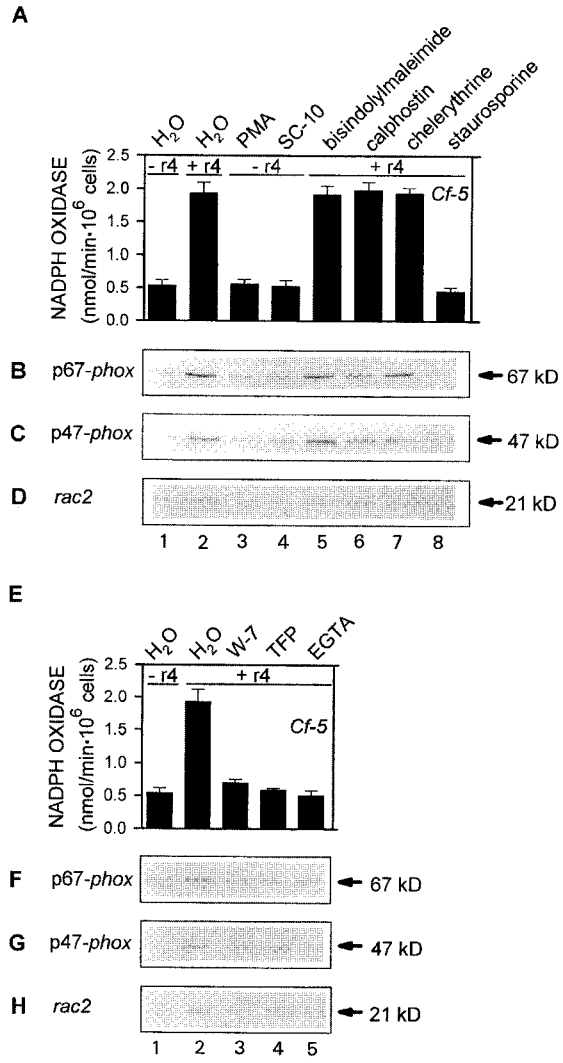

Figure 6. Effects of Kinase Activators and Inhibitors, CaM Antagonists, and EGTA on NADPH Oxidase Activity and Immunodetection of p67-*phox*, p47-*phox*, and rac2.

(A) to **(D)** *Cf-5* cells were treated for 30 min with H_2O (control), race 4 (r4) IF, 10 μM SC-10, and 250 nM PMA (lanes 1 to 4, respectively, in **[B]** to **[D]**), or with race 4 IF for 30 min but with the addition of 20 nM bisindolylmaleimide, 100 nM calphostin C, 1 mM chelerythrine, or 1 mM staurosporine 15 min before the addition of race 4 IF (lanes 5 to 8, respectively, in **[B]** to **[D]**). After plasma membrane isolation, the samples were assayed for NADPH oxidase activity **(A)**, or separated by SDS-PAGE and detected by immunoblotting with antibodies raised against p67-*phox* **(B)**, p47-*phox* **(C)**, and rac2 **(D)**.

(E) to **(H)** *Cf-5* cells were treated for 30 min with H_2O (control) and race 4 IF (lanes 1 and 2, respectively, in **[F]** to **[H]**), or with race 4 IF for 30 min but with the addition of 70 μM W-7, 100 μM TFP, or 1 mM EGTA 15 min before the addition of race 4 IF (lanes 3 to 5, respectively, in **[F]** to **[H]**). After plasma membrane isolation, the samples were assayed for NADPH oxidase activity **(E)**, or separated by SDS-PAGE and detected by immunoblotting with antibodies raised against p67-*phox* **(F)**, p47-*phox* **(G)**, and rac2 **(H)**.

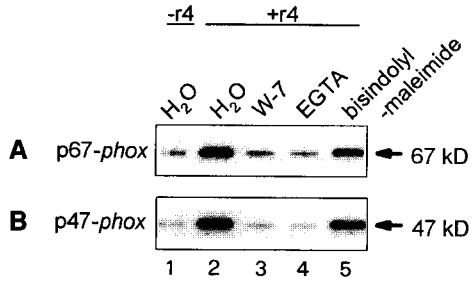

Figure 7. In Vivo γ-^{32}P-Phosphorus–Labeled Plasma Membrane Proteins Showing the Effect of W-7, EGTA, and Bisindolylmaleimide on the Phosphorylation Status of p67-*phox* and p47-*phox*.

(A) *Cf-5* cells were incubated for 6 hr with carrier-free γ-^{32}P-phosphorus followed by treatment with H_2O (lane 1, as control) and race 4 (r4) IF for 30 min (lane 2), or with race 4 IF for 30 min but with the addition of 70 μM W-7, 1 mM EGTA, or 20 nM bisindolylmaleimide 15 min before the addition of race 4 IF (lanes 3 to 5, respectively). After plasma membrane isolation and immunoprecipitation with anti–p67-*phox*, the samples were separated by SDS-PAGE.

(B) corresponds to **(A)**, except that anti–p47-*phox* antibodies were used for immunoprecipitation.

Treatment of cells with no race 4 IF is indicated by −r4. Treatment of cells with race 4 IF is indicated by +r4. The arrows represent 67- and 47-kD polypeptides that cross-reacted during immunoblotting with an antibody raised against p67-*phox* and p47-*phox* (in **[A]** and **[B]**, respectively). Gels are representative of three independent experiments.

with cytoskeleton may indicate a role of membrane skeleton in the assembly and regulation of NADPH oxidase in plants.

PKC, which is a CaM-independent protein kinase (Nishizuka, 1988; Hug and Sarre, 1993), has been shown to function in the activation and assembly of NADPH oxidase in phagocytic cells (Segal and Abo, 1993; Jones, 1994). Treatment of the cells with phorbol esters (which directly activate PKC) resulted in the activation of NADPH oxidase and phosphorylation of a number of proteins, including flavocytochrome *b* (Segal and Abo, 1993), p47-*phox* (Ding and Badwey, 1993; Ding et al., 1993; El Benna et al., 1994), and p67-*phox* (Dusi and Rossi, 1993). In tomato cells, PKC activators (PMA and SC-10) did not activate the oxidase in the absence of elicitors from incompatible *C. fulvum* races, and PKC inhibitors (bisindolylmaleimide, calphostin C, and chelerythrine) did

Treatment of cells with no race 4 IF is indicated by −r4. Treatment of cells with race 4 IF is indicated by +r4. Numbers at right represent molecular masses of protein standards in kilodaltons. Values for NADPH oxidase activities are the mean ± SE (n = 4). Calculations were based on 10^6 cells equivalent of plasma membrane proteins. Blots are representative of three independent experiments.

not inhibit the elicitor-induced activation of the enzyme. None of these activators or inhibitors affected the levels of p47-*phox*, p67-*phox*, or rac2 on the plasma membrane, although at the same concentrations, the activators enhanced and the inhibitors impeded the activity of a PKC-like kinase in the reversible phosphorylation of H^+-ATPase in the same cell line (Xing et al., 1996). In contrast, staurosporine, a general kinase inhibitor, decreased the amount of the three proteins on the plasma membrane and inhibited the elicitor-induced activation of the NADPH oxidase.

Data with PKC inhibitors agree with our previous observation showing that in tomato cells, PKC activity was not detectable during the first 30 min after elicitor treatment and increased dramatically afterward (Xing et al., 1996). In phagocytic cells, including cell-free systems, PMA was used as a model activator of NADPH oxidase (Segal and Abo, 1993; Jones, 1994). PMA was also shown to activate the production of AOS in Arabidopsis (Desikan et al., 1996). Some work in neutrophils relating to the involvement of PKC must be viewed with caution. For example, Dorseuil et al. (1995) used staurosporine as an inhibitor of PKC, which is debatable. Also, multiple activation pathways of NADPH oxidase were noted in phagocytes (Rossi, 1986). The activation of NADPH oxidase in cell-free systems may occur in the absence of PKC, and early studies also suggested that the PKC-independent pathway might involve a CaM-dependent kinase or other unknown reactions and messengers (Rossi, 1986). Different stimuli activate different proximal pathways. The response of phagocytes to PMA is Ca^{2+} independent, whereas the *f*MetLeuPhe (an example of immature peptide chains from microorganisms) response is Ca^{2+} dependent and resistant to PKC inhibitors (Segal and Abo, 1993).

The inhibitory effect of CaM antagonists (W-7 and TFP) and Ca^{2+} chelator (EGTA) suggested involvement of Ca^{2+} and CaM in the phosphorylation process in the assembly and activation of NADPH oxidase. Ca^{2+}-dependent protein kinases (CDPK) are unique protein kinases in plants that are Ca^{2+} dependent and have a CaM binding domain (Roberts and Harmon, 1992; Roberts, 1993). W-7 and TFP have been shown to inhibit the activity of CDPK (Harmon et al., 1994).

Fungal elicitor treatment also induced a rapid activation of tomato plasma membrane Ca^{2+} channels (Gelli et al., 1997) and the inhibition of the plasma membrane Ca^{2+}-ATPase (B. Lam and E. Blumwald, manuscript in preparation), elevating the net influx of Ca^{2+} ions into the cytoplasm. This increase in cytosolic Ca^{2+} concentration would contribute to the activation of the protein kinase responsible for the migration of rac, p47-*phox*, and p67-*phox* to the plasma membrane.

Using in vivo phosphorus-32 labeling, we demonstrated that elicitor treatment induced phosphorylation of p47-*phox* and p67-*phox*. The change in phosphorylation correlates with translocation of these molecules to the plasma membrane. This induced phosphorylation was significantly inhibited by EGTA or W-7 but not by bisindolylmaleimide. These observations, together with the effect of these three agents on the detectable levels of p47-*phox* and p67-*phox* on plasma membranes, indicate that the elicitor-mediated activation of NADPH oxidase in tomato cells involves phosphorylation-dependent translocation of p47-*phox* and p67-*phox* from the cytosol to the plasma membrane.

Additional evidence that the NADPH oxidase assembly process in tomato differs from that in phagocytes was our failure to detect assembly in a cell-free system. Elicitors, when added to a commonly used cell-free system in phagocytic cells (Heyworth et al., 1993) consisting of a cytosol fraction and the plasma membrane, did not affect NADPH oxidase activity or the amount of p67-*phox*, p47-*phox*, or rac2 (data not shown). "Hybrid" cell-free systems have been shown to be successful. Superoxide generation was initiated by the reconstitution of Arabidopsis plasma membranes with neutrophil cytosolic fractions but not by the combination of Arabidopsis cytosol with neutrophil plasma membranes (Desikan et al., 1996). The rupture of cells and the isolation of the plasma membrane and cytosolic fractions may affect either the conformation or the charge of p47-*phox* and/or p67-*phox* and thus block their association with the plasma membranes. It is also possible that the stability of some participating elements, such as the cytoskeleton, relies on an intact cell.

In conclusion, treatment of tomato cells with race-specific elicitors induced translocation of p67-*phox*, p47-*phox*, and rac2 from the cytosol to the plasma membranes and the activation of NADPH oxidase. The assembly process involved the phosphorylation of p47-*phox* and p67-*phox* catalyzed by a protein kinase that was Ca^{2+} dependent and inhibited by CaM antagonists. The activity of this kinase determined the translocation of p47-*phox*, p67-*phox*, and rac2 to the plasma membranes. The mechanism of NADPH oxidase in tomato cells shows high homology with the mammalian phagocyte system, suggesting that both plants and animals share common elements in eukaryotic signal transduction. Nevertheless, the involvement of different protein kinases in the activation of p47-*phox* and p67-*phox* in phagocytes and in tomato cells may reflect the unique requirements for spatial and temporal distribution in the plant cell and the differences in developmental and environmental signals to which plants must respond.

In tomato cells, fungal elicitors induce the rapid dephosphorylation of the plasma membrane H^+-ATPase (Vera-Estrella et al., 1994a; Xing et al., 1996, 1997) with the concomitant increase in H^+-pumping activity and the hyperpolarization of the plasma membrane. This results in the activation of inward Ca^{2+}-permeable channels that allow the required increase in cytosolic Ca^{2+} concentrations (Gelli and Blumwald, 1997; Gelli et al., 1997). The activation of a protein kinase other than PKC soon after elicitation impedes the premature "downregulation" by the H^+-ATPase, which is mediated by a PKC $\sim$40 min after elicitation (Xing et al., 1996). Thus, in tomato, the CDPK-mediated activation of the NADPH oxidase ensures an elicitor-induced response that is correctly timed and highly coordinated with other downstream signal transduction processes of the cells.

METHODS

Plant Material

Tomato cell suspensions derived from a line of tomato (*Lycopersicon esculentum* cv Moneymaker) carrying the resistance gene *Cf-4* or *Cf-5* were grown in 500-mL Erlenmeyer flasks containing 100 mL of Murashige and Skoog (1962) (MS) medium in the dark at 25°C on a rotatory shaker at 120 rpm and subcultured weekly (Vera-Estrella et al., 1992). Cell suspensions used in all experiments were 3 to 4 days old.

Production of Specific Elicitors

Intercellular fluids (IFs) from tomato leaf tissue infected with *Cladosporium fulvum* were prepared according to the method of De Wit and Spikman (1982). The *Cf-5*–incompatible races 2.3 and 4, or compatible races 5 and 2.4.5.9.11 (races 2.3 and 5 are incompatible on *Cf-4* plants, whereas races 4 and 2.4.5.9.11 are compatible on *Cf-4* plants), were inoculated onto the cultivar Bonny Best, which has no known *Cf* genes. The IFs were prepared as described by Xing et al. (1996). These preparations contain specific elicitors for each of the *Cf* genes on which the specific race of *C. fulvum* is avirulent (i.e., causes a hypersensitive response). In general, in the treatment of cells, a final ratio of IF to water of 1:32 was used and contained between 0.10 and 0.45 mg of protein per milliliter. The compatibility and incompatibility of IF preparations to the *Cf-5* tomato cell line have been established in various biochemical tests, including the activation of the host plasma membrane H^+-ATPase (Vera-Estrella et al., 1994a), changes in the host plasma membrane redox activities (Vera-Estrella et al., 1994b), the reversible phosphorylation of host plasma membrane H^+-ATPase (Xing et al., 1996), and the activation of trimeric G proteins (Xing et al., 1997).

In Vivo Treatment of Cells

Three- to 4-day-old cells were washed twice with activation buffer, which was as described by Dorseuil et al. (1995) as modified to contain 118 mM NaCl, 4.7 mM KCl, 25 mM Na-Hepes, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.1 μM $CaCl_2$, and 5.5 mM sucrose, pH 7.4. The cells were then suspended in a final volume of 80 mL of the same buffer. Cells were treated with IFs, protein kinase activators or inhibitors, calmodulin (CaM) antagonists, or EGTA at concentrations and for periods of time as indicated in the legend to Figure 6. Plasma membranes were then isolated from treated cells.

Plasma Membrane Isolation

Microsomal fractions from *Cf-4* and *Cf-5* cell suspensions were isolated as described previously (Vera-Estrella et al., 1994a), except that homogenization buffer contained 100 mM KCl, 3 mM NaCl, 1 mM ATP, 3.5 mM $MgCl_2$, 10 μM GTP(γ)S, 5 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 2 mM DTT, and 10 mM Pipes, pH 7.3 (Dorseuil et al., 1995). Microsomes were resuspended in 200 μL of phase suspension medium containing 250 mM sucrose, 5 mM potassium phosphate buffer, pH 7.0, and 1 mM DTT. Plasma membranes were obtained by phase partitioning microsomal preparations for three cycles in an 8- or 4-g phase system with a final composition of 6.5% (w/w) dextran T500, 6.5% (w/w) polyethylene glycol (molecular weight of 3350), 250 mM sucrose, 3 mM KCl, 5 mM potassium phosphate, pH 7.8, and 1 mM DTT (Xing et al., 1996). The phase-purified plasma membranes were either used immediately or frozen in liquid nitrogen and stored at −70°C until use. All of the above-mentioned buffers were supplemented with 1 μM staurosporine to inhibit kinase activities during membrane preparation.

Preparation of Cytosolic Fractions

Cytosolic extract was prepared using the homogenization buffer from the microsomal preparation. The homogenate was centrifuged for 60 min at 110,000*g*. The crude extract was concentrated in a concentration cell with a molecular mass cut-off of 3 kD, giving a final protein concentration of 2 to 3 mg/mL.

NADPH Oxidase Assay

Production of O_2^- by NADPH oxidase was monitored at 25°C by following the superoxide dismutase–inhibitable and NADPH-dependent reduction of cytochrome *c* at 550 nm, as described by Heyworth et al. (1993). Reaction mixtures contained 0.1 mM cytochrome *c*, 6.5 mM $MgCl_2$, 87 mM KCl, 2.6 mM NaCl, 8.7 mM Pipes, pH 7.3, 10 μM GTP(γ)S, 0.16 mM NADPH, and 10 to 15 μg of plasma membrane proteins in a total volume of 1 mL. The control assay contained 50 μg of superoxide dismutase to account for non-O_2^--dependent reduction of cytochrome *c*. Diphenylene iodonium (at 5 μM), a suicide substrate inhibitor of the phagocyte plasma membrane NADPH oxidase, inhibited the elicitor-induced activation of NADPH oxidase (data not shown).

Preparation of Membrane Cytoskeletons

Plasma membranes were incubated for 30 min at 4°C in cytoskeleton buffer (20 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 8% sucrose, 0.25 mM leupeptin, and 0.5% Triton X-100). Triton X-100–insoluble and –soluble fractions were separated by centrifugation at 180,000*g* for 2 hr at 4°C.

DNase I Treatment of Membranes

DNase I (2 mg/mL) was dissolved in cytoskeleton buffer. Membranes were treated with the DNase I–containing cytoskeleton buffer for 2 hr at 4°C. Fractions washed off with DNase I were obtained by centrifugation at 180,000*g* for 2 hr at 4°C. Control extraction was performed with cytoskeleton buffer with no additions.

SDS-PAGE and Electroblotting of Proteins

Membrane proteins were solubilized in Laemmli buffer (Laemmli, 1970). Protein (1.15 to 2.30 × 10^6 cell equivalent) separation was conducted using a discontinuous SDS-PAGE (10% separation gel) system. After electrophoresis, the proteins were transferred to nitrocellulose using a tank blotting chamber.

Protein Immunoblotting

Protein immunoblotting was performed by using antibodies against p47-*phox* (1:10,000) or p67-*phox* (1:3000) (a kind gift of P.G. Heyworth, Scripps Research Institute, La Jolla, CA). These p67-*phox* and p47-*phox* anti-peptide antibodies were raised against human protein peptide sequences 437DEPKESEKADANNQ450 and 340RPGPQSPGSPLEEERQ355, respectively. Primary antibodies against rac1 and rac2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

In Vivo ^{32}P-Phosphorus Labeling and Immunoprecipitation

Three- to 4-day-old cell suspensions were supplemented with 1 mCi of carrier-free ^{32}P-phosphorus followed by incubation for 6 hr. The cells were treated with IFs for 30 min followed by plasma membrane isolation. Plasma membranes were treated with cytoskeleton buffer for 30 min at 4°C. Triton X-100–insoluble fractions were further treated with DNase I–containing cytoskeleton buffer for 2 hr at 4°C. After centrifugation at 180,000*g* for 2 hr at 4°C, the supernatant equivalent to 15 to 25 μg of plasma membrane proteins was boiled for 5 min in 2 × immunoprecipitation buffer (20 mM sodium phosphate, pH 7.2, 300 mM NaCl, 2 mM EDTA, and 2% Triton X-100), followed by centrifugation for 10 min at 15,000*g*. An equal volume of 2 × immunoprecipitation buffer was added to the supernatant together with 25 μL of antibodies raised against p47-*phox* or p67-*phox*. After 10 min of gentle mixing, samples were incubated overnight at 4°C. An equal volume of 10% prewashed protein A–agarose was added. (Protein A–agarose was washed with 50 volumes of 1 × immunoprecipitation buffer by mixing and microcentrifugation and resuspended in immunoprecipitation buffer, giving a final concentration of 10%). The samples were incubated at room temperature for 2 hr before centrifugation for 4 min at 15,000*g*. The supernatant was discarded, and the pellet was washed three times with 1 mL of immunoprecipitation buffer by mixing and microcentrifugation. The pellet was finally resuspended in SDS-PAGE sample buffer (Laemmli, 1970) and centrifuged at 15,000*g*, and the supernatant was electrophoresed on an SDS–polyacrylamide gel (10% separation gel). The pattern of phosphorylated polypeptides was visualized by autoradiography by using Kodak X-Omat AR autoradiography film.

Protein Determination

Protein was measured according to Bradford (1976), with BSA as standard.

Chemicals

Bisindolylmaleimide, calphostin C, chelerythrine chloride, phorbol 12-myristate 13-acetate (PMA), staurosporine, and trifluoperazine (TFP) dimaleate were obtained from CalBiochem (San Diego, CA). W-7 (*N*-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide, HCl) and SC-10 (*N*-heptyl-5-chloro-1-naphthalenesulfonamide) were purchased from Sigma. γ-^{32}P-phosphorus was purchased from Amersham International. Other common chemicals were purchased either from ICN Biomedicals (Mississauga, Ontario, Canada) or BDH Inc. (Toronto, Ontario, Canada).

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