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TEMPORAL REGULATION OF TOBACCO MOSAIC VIRUS-INDUCED PHOSPHORYLATION OF A HOST ENCODED PROTEIN

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Summary. The *in vitro* and *in vivo* phosphorylation of a plant encoded protein (p68) associated with dsRNA-dependent protein kinase activity was stimulated at specific time intervals following infection by tobacco mosaic virus or electroporation with dsRNA. The level of p68 phosphorylation in infected and mock inoculated protoplasts did not differ significantly until 6 hr. post-infection, when the basal level of phosphorylation increased 2-3 fold in infected protoplasts. Maximum phosphorylation of p68 occurred between 8-12 hr post-infection and then declined but, at least until 72 hr. post-infection, it was significantly greater than in mock inoculated protoplasts.

Successful viral pathogenesis is dependent upon the efficient utilization of host proteins to complement and extend the limited genomic capacity of the virus. This requires a regulatory system capable of altering the function of endogenous host proteins during key phases of pathogenesis.

Nucleotide binding and protein phosphorylation are key post-translational events which provide an efficient mechanism to amplify the cellular response rate to various extracellular signals. The functional significance of phosphorylation-mediated regulatory cascades, especially in plant systems and during plant-pathogen interactions, are only partly understood (1,2).

Recently, we identified and partially characterized a plant encoded Mr 68,000 (p68) phosphoprotein associated with dsRNA dependent protein kinase activity (3). The plant encoded p68 was isolated from leaf tissues infected with TMV for 10-14 d when severe mosaic symptoms were visible. In TMV infected leaf tissue only 0.1% of cells are initially infected (4) and secondary infections make precise kinetic evaluations difficult. Identification of specific signals regulating viral-host responses requires a system where very early events can be analyzed. Protoplasts from leaves or cell cultures can be synchronously infected and have proven useful in defining the temporal progression of virus infection. In addition, because high infection percentages are possible in protoplast systems, functional assays can be done without a high background of uninfected cells.

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Here we demonstrate that phosphorylation of the Mr 68,000 host encoded protein, *in vitro* and *in vivo*, is regulated in a time-specific manner in response to virus infection, synthetic dsRNA or TMV dsRNA.

MATERIALS AND METHODS

Culture and Inoculation of Protoplasts. Protoplasts were isolated from tobacco (*Nicotiana tabacum* L. cv. Samsun nn) according to Bornman (5). The condition of the protoplasts was evaluated based upon membrane integrity and chloroplast appearance. Percent protoplast viability was determined using triplicate aliquots by fluorescein diacetate staining (6).

TMV virions (U_1 strain) and TMV dsRNA were purified from infected tobacco according to Asselin and Zaitlin (7) and Morris and Dodds (8), respectively. TMV RNA was purified from virions according to Bruening et al. (9). Tobacco mRNA was purified from healthy plant tissue according to Sambrook et al. (10). Protoplasts were infected with TMV virions (2 μg/ml) by adding the inoculum in 0.02 M potassium citrate, pH 5.2, 0.4 M mannitol, and 1.6 μg/ml polyethyleneimine to washed protoplasts (5 x 10^6/ml), mixing gently for 10 min followed by washing three times in 0.4 M mannitol. Protoplasts were then resuspended in Takebe's medium (11) and incubated for times between 0-72 hrs at 25° under low light. Control treatments of mock inoculated protoplasts were treated identically to infected treatments throughout the experiments.

Electroporation of viral RNAs, synthetic dsRNAs and other nucleic acids into protoplasts was done by subjecting protoplasts (1 ml sample containing 5 x 10^6 protoplasts) to a single 150 msec discharge at 240 V and 120 μF in a Bio-Rad Gene Pulser apparatus in the presence of the nucleic acids. Following electroporation, the protoplasts were incubated at 0° for 10 min, washed three times in 0.4 M mannitol and resuspended in Takebe's medium for times described in the results.

The percent infection was determined by indirect immunofluorescent staining of protoplast aliquots following 24 hr. incubation using goat anti-TMV coat protein serum obtained from Agdia, Inc. (Mishawaka, Indianna) and protein A-FITC (Sigma). We routinely performed immunofluorescence analyses in triplicate on slides containing approximately 1 x 10^6 cells. Samples were viewed under a Nikon Labophot microscope equipped for fluorescence microscopy.

In vitro phosphorylation assays. Protoplasts were washed 5 times in ice-cold 0.4 M mannitol and homogenized in E buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 2 mM $MgCl_2$, 0.2 mM PMSF, 1% Triton X-100). Extracts were filtered through Miracloth and centrifuged 2 hr at 125,000 x g to yield a S100 post-ribosomal fraction. Protein concentrations were determined using a Pierce assay kit (Pierce Chemical Co.). *In vitro* phosphorylation assays and product analysis were done according to (12, 13).

In vivo phosphorylation analyses. Washed protoplasts (2 x 10^6 cells/ml) were mock inoculated or inoculated with TMV virions (2 μg/ml) as described above and incubated in Takebe's medium. Following incubation for 1, 5 and 9 hrs., aliquots containing 1 x 10^6 cells were washed with 0.4 M mannitol and transferred to Takebe's medium lacking phosphate. Following 1 hr. incubation, 100 μCi $H_3{}^{32}PO_4$ (specific activity = 285 Ci/mg P) was added and incubation continued for 2 hr. Fractions, enriched for p68 and obtained as described below, were subject to SDS-polyacrylamide gel electrophoresis and autoradioradiography according to (12,13).

RESULTS

The level of p68 phosphorylation in extracts from TMV infected protoplasts was similar to mock treatments until 6 hr post-inoculation when stimulation was first observed (data not shown). Phosphorylation was significantly stimulated at 8 hr post-infection (Fig. 1, Lane 5) and reached a maximum, 4-5-fold higher than comparable mock inoculated treatments, 12 hr post-infection (Fig. 1, Lane 7). The stimulation subsequently declined but was maintained at a steady state level 1.5-2-fold greater than in mock inoculated treatments until 72 hr. When the mean percent infection of protoplasts is accounted for, phosphorylation of p68 in infected protoplasts was stimulated at 12 hr. post-infection at least 6-8 fold by TMV infection. The level of p68 phosphorylation in extracts from mock inoculated protoplasts was uniform throughout the incubation schedule.

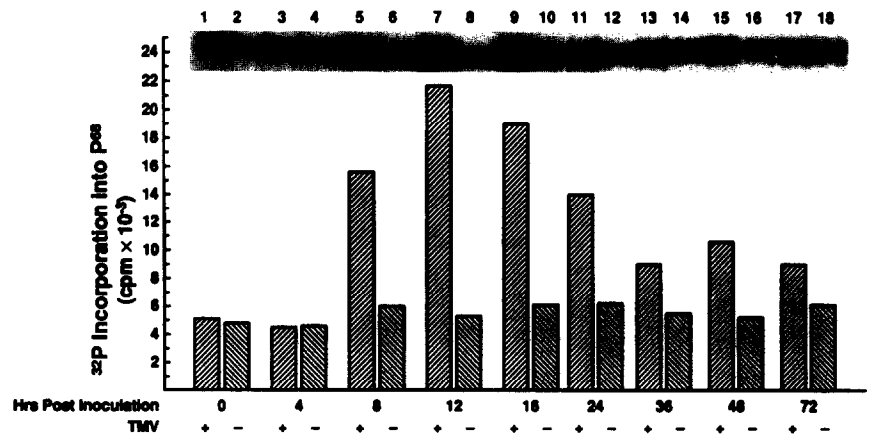

Figure 1. **Temporal regulation of p68 phosphorylation by TMV infection.** Tobacco protoplasts were synchronously infected with TMV or mock inoculated and incubated for 0, 4, 8, 12, 16, 24, 36, 48, or 72 hrs. Fractions enriched for p68 were subjected to *in vitro* phosphorylation assays, followed by SDS-polyacrylamide gel electrophoresis and autoradiography. Quantification of ^{32}P incorporation from (g ^{32}P) ATP into p68 was done by Cerenkov counting of gel slices and confirmed by densitometer scanning of the autoradiogram. The autoradiogram is of the Mr 68,000 region of the gel.

Phosphoamino analysis of the doublet at Mr 68,000 indicates that the bands are of the same species (He, et al., manuscript in preparation). Protoplast viability, based upon fluoroscein diacetate staining, was between 95 and 98% from 0 time until 36 hr post-incubation. Between 48 and 72 hr incubation, the viability decreased to 75%. Incubation of protoplasts with TMV virions (2 μg/ml) in the presence of polyethyleneimine consistently resulted in greater than 60% infection. This was based upon counting protoplasts following immunofluorescence staining 24 hr post-infection.

The phosphorylation of p68 was also monitored in extracts from protoplasts electroporated in the presence of TMV RNA, poly I, poly I-poly C, TMV dsRNA isolated from infected plants, and mRNA from healthy plants. The temporal response of p68 phosphorylation to electroporation of protoplasts with TMV RNA at 15 μg/ml was essentially similar to that observed in extracts of protoplasts infected with TMV virions (data not shown). Peak stimulation between 8-12 hr post-electroporation was 4-5 fold higher than in mock inoculated treatments or controls consisting of electroporation plus, nucleic acid minus treatments. No significant differences were found in p68 phosphorylation between minus nucleic acid or minus electroporation treatments and in treatments where activity was assayed immediately following electroporation with TMV dsRNA (15 μg/ml), poly I-poly C (50 μg/ml), or poly I (50 μg/ml)(Fig. 2, lanes 1-5). However, at 8 hr post-electroporation with TMV dsRNA or poly I-poly C, p68 phosphorylation was significantly increased compared to minus nucleic acid or minus electroporation treatments (Lanes 6, 7 vs. 8, 10). Electroporation with 50 μg/ml poly I did not affect the activity of p68 (Lane 9). Other concentrations of poly I (15, 25, 100, 150 μg/ml) were also electroporated into protoplasts with no affect on the level of p68 phosphorylation (data not shown). Essentially similar results were obtained when incubation was continued to 12 hr. post electroporation (Lanes 11-15). When TMV dsRNA (10μg/ml) was added to extracts of mock inoculated protoplasts during *in vitro* phosphorylation (Lane 16), the level of p68 phosphorylation was similar to that found in Lanes 6, 7, 11, 12. P68 phosphorylation in protoplasts was not stimulated above basal levels by

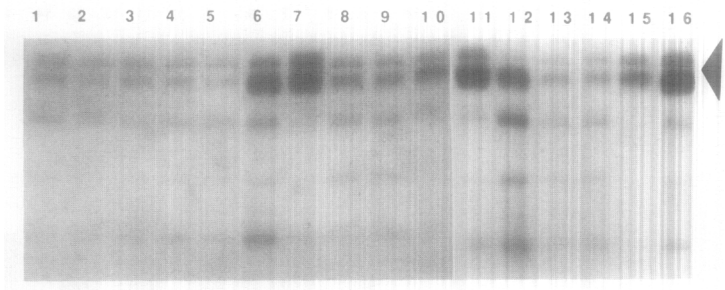

Figure 2. **Regulation of p68 phosphorylation by electroporation of TMV dsRNA or poly I-poly C.** TMV dsRNA (15 µg/ml), isolated from TMV infected tissues according to Morris and Dodds (8), poly I-poly C (50 µg/ml) or poly I (50 µg/ml) were electroporated into protoplasts. Protoplasts were incubated for 0 (Lanes 1-5), 8 (Lanes 6-10) and 12 (Lanes 11-15) hrs prior to partial purification of p68, *in vitro* phosphorylation, SDS-polyacrylamide gel electrophoresis and autoradiography. In the treatment shown in Lane 16, TMV dsRNA (10 µg/ml) was added to mock inoculated lysates during *in vitro* phosphorylation. The autoradiogram shown here was exposed for 4 hr at -80°. Arrow indicates position of p68.

electroporation in the presence of mRNA (10, 25, 50 and 100 µg/ml) from healthy tobacco leaves. In addition, electroporation in the presence of TMV virions induced p68 phosphorylation similarly to that found for polyethyleneimine-mediated virion infection as described above (data not shown).

The response of p68 to dsRNA was concentration dependent. Sub-optimal levels of electroporated TMV dsRNA (< 5 µg/ml) or poly I-poly C (<25 µg/ml) were not stimulatory to p68 phosphorylation. However, significant stimulation over basal levels found in minus electroporation treatments was found with TMV dsRNA at 10µg/ml (Fig. 3). Electroporation of higher concentrations of poly I-poly C (50 µg/ml) was necessary to obtain p68 phosphorylation

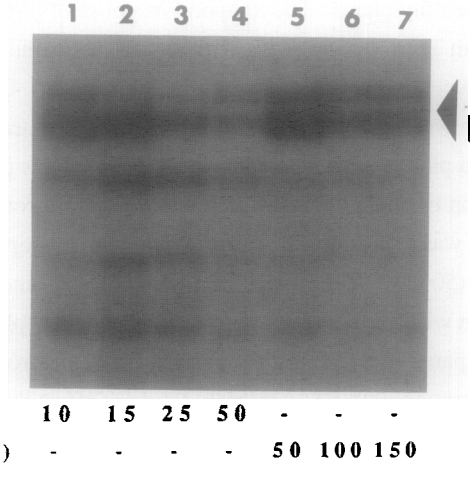

Figure 3. **Phosphorylation of p68 is dependent upon the concentration of dsRNA electroporated into protoplasts.** Varying concentrations of TMV dsRNA and poly I-poly C were electroporated into protoplasts and incubated for 1 hr. Lysates were subject to *in vitro* phosphorylation assays, SDS-polyacrylamide gel electrophoresis and autoradiography. The autoradiogram shows the Mr 68,000 region of the gel. Quantification of ^{32}P into p68 was done by Cerenkov counting of gel slices and confirmed by densitometer scanning of the autoradiogram. Arrow indicates position of p68.

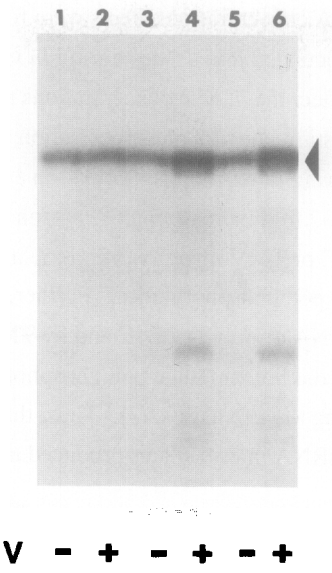

Figure 4. ***In vivo* phosphorylation of p68 is induced by TMV infection.** Protoplasts were mock inoculated (Lanes 1, 3, 5) or TMV infected (Lanes 2, 4, 6) and pulse labelled with (^{32}P) H_3PO_4 for 2 hr following 2 (Lanes 1, 2), 6 (Lanes 3, 4) and 10 (Lanes 5, 6) hr incubation. Protoplasts were then lysed and p68-enriched fractions were subject to SDS-polyacrylamide gel electrophoresis and autoradiography. The autoradiogram was exposed for 48 hr at -80°. Arrow indicates position of p68.

comparable to electroporation with TMV dsRNA (10μg/ml). At 25 μg/ml and >100 μg/ml for TMV dsRNA and poly I-poly C, respectively, p68 phosphorylation was significantly depressed relative to peak stimulation levels. Percent protoplast viability in all treatments in Figures 2, 3 was approximately 95% until 24 hr. At 36 hr viability decreased to 80%. Percent protoplast infection by electroporation of either TMV RNA or virions was approximately 70% based upon immunofluoresence staining at 24 hr post-electroporation.

Phosphorylation of p68 *in vivo* was also examined in response to TMV infection. Figure 4 shows that the basal level of p68 phosphorylation in protoplasts significantly increases (4-6 fold) between 6-8 hr (Lane 4) and 10-12 hr (Lane 6) by infection with TMV. Phosphorylation of p68 was similar in mock inoculated protoplasts throughout the experiments.

DISCUSSION

Plant virus-host interactions are clearly based upon elaborate regulatory mechanisms which temporally mediate host and pathogen metabolic processes. We have shown that the phosphorylation of a plant encoded protein is temporally regulated by TMV infection and associated with dsRNA dependent protein kinase activity. This was demonstrated with both *in vitro* and *in vivo* assays. Previous studies suggested that p68 itself may have dsRNA-dependent kinase activity, but the temporal nature of the response was not shown (3, 12). An analogous enzyme has been well characterized from mammalian systems (14, 15).

Evidence suggests that both plant and animal p68 phosphorylation is mediated by infective viral RNA, dsRNA intermediates or dsRNA (16). Regulation of p68 phosphorylation in the plant system is dependent upon the concentration of dsRNA. Stimulation occurs in the range of 10-15 μg/ml TMV dsRNA and levels less than 5 μg/ml or greater than 25 μg/ml dsRNA inhibit p68

phosphorylation. Similar dsRNA concentration effects were found when the TMV dsRNA was added to extracts from mock inoculated leaf tissues during *in vitro* phosphorylation assays (3). These levels may not exactly reflect the *in vivo* concentrations necessary to regulate p68 activity. Further, TMV ssRNA does not stimulate p68 phosphorylation when added to extracts from mock inoculated tissues prior to or during *in vitro* phosphorylation assays. Under the same conditions, TMV dsRNA elicts, at least, a 2-3 fold increase in p68 phosphorylation. Similarly, electroporation of TMV dsRNA or poly I-poly C or TMV infection of protoplasts induces the same qualitative and quantitative response relative to p68 phosphorylation. Further, this response mimics that observed for electroporation of TMV dsRNA or poly I-poly C and by TMV infection. Exogenous mRNA, rRNA, poly I or total host RNA did not stimulate p68 phosphorylation when electroporated into protoplasts or when added during *in vitro* assays (3). Thus, the induction of p68 phosphorylation by TMV is most likely due to dsRNA intermediates produced at specific times during pathogenesis.

Several factors support the concept that phosphorylation is a direct result of pathogen infection. Most importantly, p68 phosphorylation is regulated at specific times during the infection cycle and phosphorylation responds to signal strength. P68 phosphorylation in protoplasts is correlated with the temporal pattern of synthesis of TMV RNAs and proteins (17, 18). In general, TMV RNAs (dsRNA, genomic RNA, 30 Kd mRNA and coat protein mRNA) and the corresponding protein products are initially detectable between 3-6 hr post-infection. Production of dsRNA, genomic RNA and coat protein mRNA and their proteins increase exponentially until 12-16 hr at which time the level of synthesis plateaus but is maintained until at least 48 hr post-infection.

A putative function of p68 phosphorylation, based upon analogous models, is in the regulation of protein synthesis (19, 20). Fraser and Gerwitz (21) found that in TMV infected tobacco, protein synthesis was reduced by up to 75% during rapid virus multiplication but later recovered. Further experiments are underway to determine if plant p68 phosphorylation is involved in the regulation of protein synthesis in virus infected and in healthy plants.

The temporal regulation of p68 phosphorylation is an early event in TMV pathogenesis *in vitro* and *in vivo*. The phosphorylation is associated with dsRNA-dependent protein kinase activity which specifically responds in a concentration dependent fashion to TMV dsRNA suggesting a role in signal transduction.

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