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Viroid-Induced Phosphorylation of a Host Protein Related to a dsRNA-Dependent Protein Kinase

H. J. HIDDINGA, C. JESSEN CRUM, JIE HU, D. A. ROTH*

Viroids are very small, unencapsidated RNAs that replicate and induce severe disease in plants without encoding for any proteins. The mechanisms by which the viroid RNA regulates these events and interacts with host factors are unknown. An M_r 68,000 host-encoded protein has been identified that is differentially phosphorylated in extracts from viroid-infected and mock-inoculated tissues. This phosphoprotein is immunologically related to a double-stranded (ds) RNA–dependent protein kinase from virus-infected, interferon-treated human cells. Further, nucleotide photoaffinity labeling indicates that the protein has an ATP binding site. This protein is similar to dsRNA-dependent protein kinases implicated in mammalian systems in the regulation of protein synthesis and virus replication.

VIROIDS COMPRISE A DISTINCT AND unique class of pathogenic agents consisting of single-stranded RNA molecules of between 250 and 400 nucleotides with a high degree of base pairing (*1*). They replicate autonomously in plants, lack any detectable mRNA activity, and have no protective capsid (*1, 2*). Significant progress has been made in determining the structure and nucleotide sequence of many viroids. Relatively little is known, however, regarding possible mechanisms of pathogenicity (*2*). Viroids replicate and induce severe disease in plants without encoding for any proteins. This suggests that the viroid RNA may interact with selected host factors to regulate replicative processes and initiate events culminating in symptom expression. Identification of viroid-associated host factors and the characterization of their role in normal and infected plants is an area of great interest but one in which little progress has been made.

Experiments presented here provide evidence for a viroid-induced alteration of a host-encoded protein. We have found that potato spindle tuber viroid (PSTV) infection of tomato plants selectively alters the phosphorylation state of an M_r 68,000 host-encoded protein (designated p68). Furthermore, several lines of evidence support the notion that this phosphoprotein has dsRNA-dependent protein kinase activity.

To determine if viroid infection alters the phosphorylation state of host cell proteins, we prepared mock-inoculated and PSTV-infected tomato tissues and dialyzed as described (*3*). Homogenates from PSTV-infected and mock-inoculated tissues were treated identically, and exactly equal aliquots of each were loaded onto polyacrylamide gels. Densitometer tracings of the Coomassie blue–stained gels indicated that equal amounts of protein were present in homogenates from PSTV-infected and mock-inoculated tissues. The ^{32}P incorporation from [γ-^{32}P]ATP into two bands in the region of M_r 68,000 was significantly elevated in extracts from PSTV-infected plants as compared to mock-inoculated plants (Fig. 1, lanes 1 and 2). This autoradiogram was exposed for only 2 hours, and the only labeled bands present were in the region of M_r 68,000. Longer exposure revealed other labeled peptides; however, under these conditions the doublet could not be resolved well. The doublet bands seen appear to be the result of differential phosphorylation of a single p68 protein, since label from the lower band could be chased into the higher band by addition of cold ATP. Petryshyn *et al.* (*4*) observed a similar phosphorylation pattern with the dsRNA-dependent protein kinase from reticulocyte lysates. Densitometer scans of the autoradiogram in Fig. 1 indicate that in homogenates from infected plants approximately threefold as much ^{32}P is incorporated into the p68 bands as into homogenates from mock-inoculated plants. Phosphorylation of p68 in tomato tissue homogenates was dependent on Mg^{2+}, stimulated by Mn^+, and independent of cAMP and cGMP. In addition, [γ-^{32}P]8-azido adenosine triphosphate (8-N_3ATP) mimicked [γ-^{32}P]ATP in phosphotransferase activity.

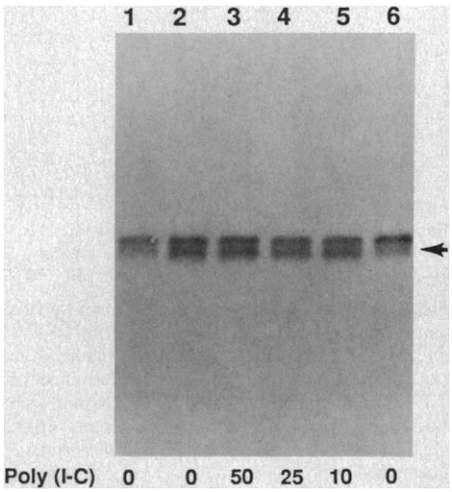

Fig. 1. Incorporation of ^{32}P from [γ-^{32}P]ATP into p68 from homogenates of PSTV-infected (lane 2) and mock-inoculated (lanes 1, 3, 4, 5, and 6) tissues. Homogenates (*3*) were incubated with 1 μM [γ-^{32}P]ATP (100 Ci/mmol), 20 mM tris-HCl (*p*H 7.4), 5 mM $MgCl_2$, 5 mM $MnSO_4$, and dsRNA at concentrations indicated for 10 min at 30°C. Protein solubilizing mix (*3*) was added and the mixture was heated in a boiling water bath for 4 min. Polypeptides were separated on a 10% polyacrylamide gel containing SDS, which was then fixed, stained with Coomassie blue, and autoradiographed. Arrowhead shows p68.

Department of Plant, Soil, and Insect Sciences, University of Wyoming, Laramie, WY 82071.

*To whom correspondence should be addressed.

It was also possible to alter the level of p68 phosphorylation in extracts from mock-inoculated tissues by adding dsRNA during phosphorylation treatments to levels comparable with those in extracts of PSTV-infected tissue. This increased phosphorylation in the presence of dsRNA was specific for p68; the level of ^{32}P incorporation into other proteins in the preparation was not influenced by exogenous dsRNA. Although dsRNA (1 μg/ml) increased phosphorylation of p68, maximum stimulation was achieved with 10 to 50 μg of exogenous dsRNA per milliliter in crude homogenates under our conditions. At concentrations of synthetic dsRNA >50 μg/ml the stimulatory effect was reversed.

Similar alterations in the levels of phosphorylation in the presence of dsRNA have also been observed for an M_r 67,000 to 71,000 protein (designated P_1) from virus-infected, interferon-treated, mammalian cells (*5*). This protein is an autophosphorylating dsRNA-dependent protein kinase (*6, 7*). The similarity between p68 and P_1 raised the interesting possibility that p68 may have analogous kinase activity. In homogenates from PSTV-infected tissues radiolabeled p68 was immunoprecipitated by antiserum to P_1 (anti-P_1) but not by nonimmune sera or no sera (Fig. 2). This indicates that the plant p68 has immunological as well as physiological similarities to the mammalian P_1 protein. Phosphorylated p68 was also immunoprecipitated from homogenates from mock-inoculated tissue. When dsRNA (50 μg/ml) was added to extracts from mock-inoculated tissues during phosphorylation reactions, then followed by immunoprecipitation as described above, concentrations of ^{32}P-labeled p68 increased over basal levels from mock-inoculated tomato tissues.

Photoaffinity labeling with azido analogs of nucleotides has been successfully used to identify nucleotide binding proteins in crude tissue homogenates (*3, 8*). In particular, 8-NA_3ATP specifically labels the nucleotide binding site of proteins with phosphotransferase activity (*8*). We utilized [α-^{32}P]8-NA_3ATP to determine if p68 has a nucleotide binding site consistent with characteristics known for protein kinases. Photolabeling was dependent on ultraviolet (UV) illumination and Mg^{2+} but not cAMP. Photoincorporation of 8-NA_3ATP into p68 was decreased by addition of 200 μ*M* ATP but not CTP, GTP, or UTP (Fig. 3). Further, the photolabeled M_r 68,000 protein can be immunoprecipitated with anti-P_1. These data indicate that p68 does have a nucleotide binding site and that this site has the highest affinity for ATP; these are characteristics observed for other protein kinases (*8*).

That p68 has protein kinase activity is further suggested by the ability of anti-P_1 immunoprecipitates from extracts of PSTV-infected tissues to phosphorylate endogenous p68. For these experiments, tissue homogenates were incubated with antiserum, normal serum, or no serum as described above. The immunocomplex was collected by centrifugation, washed thoroughly, solubilized in 20 m*M* tris-HCl (*p*H 7.4), 5 m*M* $MgCl_2$, and 5 m*M* $MnSO_4$ and then incubated with 1 μ*M* [γ-^{32}P]ATP (100 Ci/mmol). Immunoprecipitates from PSTV-infected extracts catalyzed the phosphorylation of endogenous p68 (Fig. 4). These data suggest either that p68 has kinase activity or that a very closely associated kinase that immunoprecipitates with p68 is present. Although the latter possibility exists, results from photoaffinity labeling experiments support the notion that p68 is the protein kinase.

These experiments provide evidence for a PSTV-induced functional alteration of a host-encoded protein. We have identified a similar p68–protein kinase complex in other plant virus–host systems, suggesting a possible conserved activity between viruses and

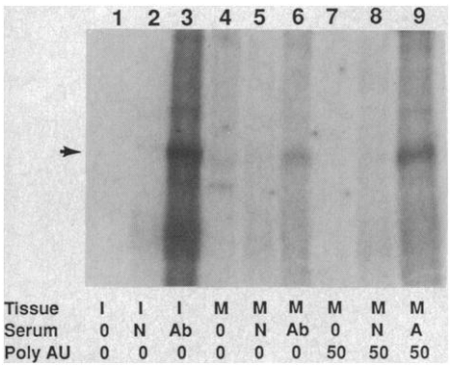

Fig. 2. Immunoprecipitation of p68 from tomato tissue homogenates. Homogenates from PSTV-infected and mock-inoculated tomato tissues were phosphorylated in the presence or absence of dsRNA as described in Fig. 1 and incubated with anti-P_1 serum (1:40 dilution), normal serum (1:40 dilution), or no serum for 12 hours at 4°C. Formalin-fixed *Staphylococcus aureus* Cowan 1 strain cells (3%, v/v) were added, and incubation was continued for 45 min at 4°C. The pellet from a 12,000*g* centrifugation containing the immunocomplex was washed three times in cold STN buffer (*7*) followed by solubilization (*3*) and heating for 4 min in a boiling water bath. Supernatants from a further centrifugation were loaded on a 10% polyacrylamide gel containing SDS. The autoradiogram is shown here. Arrowhead shows p68. I, homogenate from PSTV-infected tissues; M, homogenate from mock-inoculated tissues. 0, no serum added; N, normal serum; Ab, anti-P_1 serum. Poly AU: 0, none, or 50 for 50 μg/ml.

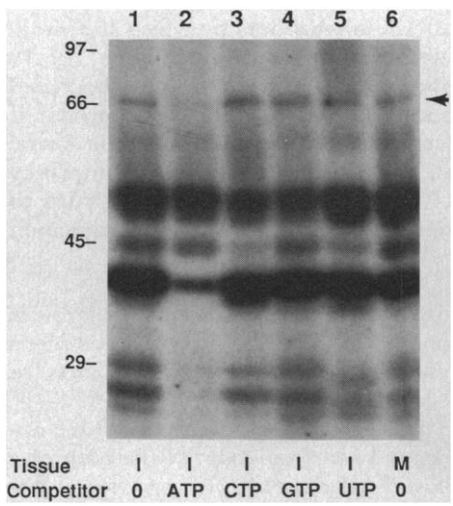

Fig. 3. Nucleotide photoaffinity labeling of tomato tissue homogenates. Homogenates from PSTV-infected and mock-inoculated tissues were incubated for 10 s at 4°C in the presence of 20 μ*M* [γ-^{32}P]8-NA_3ATP (8 Ci/mmol) in 20 m*M* tris-HCl (*p*H 7.4) and 5 m*M* $MgCl_2$ in the absence (lanes 1 and 6) or presence (lanes 2 to 5) of competitors. Nonradioactive competitors were added to a final concentration of 200 μ*M*. Samples were irradiated (254 n*M* UVS-11 Mineralight, 640 μW/cm^2) at a distance of 1 cm for 1 min. Mixtures were immediately solubilized and subjected to SDS–polyacrylamide gel electrophoresis and autoradiography (*3*). The autoradiograph is shown here. Arrowhead shows p68. Molecular weight markers × 10^3 are shown at the left of lane 1. I, homogenates from PSTV-infected tissues; M, homogenates from mock-inoculated tissues.

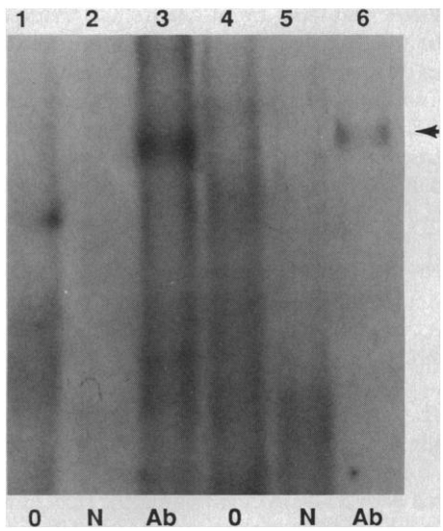

Fig. 4. Protein kinase activity associated with the p68 immunocomplex. Dialyzed, nonphosphorylated homogenates from PSTV-infected (lanes 1, 2, and 3) and mock-inoculated (lanes 4, 5, and 6) tissues were immunoprecipitated and washed as described in Fig. 2. The washed immunocomplex was incubated with 20 m*M* tris-HCl (*p*H 7.4), 5 m*M* $MgCl_2$, 5 m*M* $MnSO_4$, and 1 μ*M* [γ-^{32}P]ATP (100 Ci/mmol) for 10 min at 30°C. Protein solubilization mix (*3*) was added and the mixture heated for 4 min in a boiling water bath. Supernatants from a 12,000*g* centrifugation were subjected to SDS–polyacrylamide gel electrophoresis and autoradiography as described in Fig. 1. Arrowhead shows p68. 0, no serum added; N, normal serum; Ab, anti-P_1 serum.

viroids. In homogenates from tobacco mosaic virus–infected tobacco, brome mosaic virus–infected barley, and cowpea mosaic virus–infected cowpeas ^{32}P-incorporation from [γ-^{32}P]ATP into two bands in the region of M_r 68,000 was significantly elevated compared to extracts from the respective mock-inoculated tissues. Nucleotide photoaffinity labeling and immunoprecipitation experiments showed that the M_r 68,000 protein in these systems has an ATP-binding site and is immunologically related to the P_1 dsRNA-dependent protein kinase. Nucleotide-protein interactions that involve nucleotide binding and protein phosphorylation have been implicated in the regulation of metabolic events in diverse mammalian cell–virus interactions (*10–14*). Although the role of phosphorylation in viroid replication or pathogenesis is not clear, several possibilities exist. One potential function, in both normal and infected cells, is in the regulation of protein synthesis. A dsRNA-dependent protein kinase is found in reticulocyte lysates and regulates the initiation of translation by interfering with the recycling of the eIF-2/GTP/Met-tRNA ternary complex (*16*). The only known physiological substrate for this enzyme and the P_1 dsRNA-dependent kinase from interferon-treated cells is the α subunit of eIF-2 (*7, 10*). Recently, Kitajewski *et al.* (*15*) have shown that induction of P_1 kinase activity is associated with the inhibition of protein synthesis.

Data presented by Morrow *et al.* (*12*) suggest that the host factor necessary for poliovirus replication is an M_r 67,000 phosphoprotein associated with dsRNA-dependent protein kinase activity. The α subunit of eIF-2 is a substrate for this enzyme (*12*). We have also shown that an M_r 68,000 host-encoded protein from tobacco is phosphorylated in crude tobacco mosaic virus replicase preparations, suggesting a possible role in replication in that system (*17*).

Evidence has been provided to support a model for viroid replication that involves direct RNA to RNA copying via a rolling circle mechanism; replication would be mediated by a host polymerase (*18*). RNA species in the replication complex have been analyzed but associated proteins have not yet been identified (*19*). As viroids encode no polypeptides they must manipulate host cells in a way to obtain components necessary for their replication. Analysis of interactions between viroid RNA (and intermediates found in the replication cycle) and host factors can suggest possible mechanisms of regulation. The requirement of dsRNA in vivo may be met by highly intramolecularly base-paired single-stranded RNA or by dsRNA replicative intermediates. Protein synthesis in reticulocyte lysates is inhibited by dsRNA with as few as 50 matching base pairs, and there is no specificity relative to base composition or base sequence (*20*). The hairpin-like structure of poly(A)–poly(U) is apparently sufficient structure for recognition. Samuel (*10*) found that reovirus single-stranded RNA stimulates the phosphorylation of P_1 and eIF-2. In this regard, viroid RNA as covalently closed circles may be adequate for activation of the p68 kinase. At this time we cannot exclude the possibility that the effect of PSTV infection on p68 may be a secondary event, not directly relevant to replication or viroid pathogenesis. These possibilities will be resolved, in part, by purification of the protein and evaluation of the effect of viroid RNA species on enzyme activity.

In conclusion, we have identified a viroid-induced phosphorylation of a host-encoded protein. This protein has kinase activity or is closely associated with such activity. The role of this protein in viroid pathogenesis is presently unclear, but possibilities include the regulation of protein synthesis and replication.

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Fish Oils Inhibit Endothelial Cell Production of Platelet-Derived Growth Factor–Like Protein

PAUL L. FOX* AND PAUL E. DICORLETO

Diets rich in fish and fish oils are associated with a reduced risk of cardiovascular disease and atherosclerosis. The interaction of a commercial fish oil extract (MaxEPA) with vascular endothelial cells (ECs) was studied as a possible mechanism for this protective effect. MaxEPA almost completely inhibited EC production of platelet-derived growth factor–like protein (PDGFc) while other lipids had a lesser effect or no effect. Overall protein synthesis was not reduced, nor was the inhibition due to defective secretion or increased degradation of the growth factor. Antioxidants suppressed the inhibitory activity of MaxEPA indicating that free radical oxidative processes were required for the inhibition. These results suggest that fish oils may suppress intimal smooth muscle cell proliferation by decreasing the production of EC-derived paracrine growth factors. This inhibitory process represents a possible molecular mechanism for the antiatherosclerotic action of marine lipids.

FISH OILS ARE HETEROGENEOUS MIXtures of lipids, primarily triglycerides, containing an abundance of two unusual polyunsaturated fatty acids, eicosapen-

Department of Brain and Vascular Research, Cleveland Clinic Research Institute, Cleveland, OH 44195.

*To whom correspondence should be addressed.