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Two antiviral proteins from tobacco: Purification and characterization by monoclonal antibodies to human β-interferon

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ABSTRACT **Polyclonal antibodies to human β-interferon reacted specifically with two plant proteins (gp22 and gp35) by Western blot analysis of crude protein extracts from tobacco leaves infected with tobacco mosaic virus. Immunoaffinity chromatography of these extracts on a column of immobilized monoclonal antibodies to human β-interferon and then reversed-phase HPLC yielded gp22 and gp35 in a pure state. Both proteins reacted with the Schiff reagent and concanavalin A (indicating their glycoprotein nature) and exhibited antiviral activity (inhibiting tobacco mosaic virus replication in tobacco-leaf discs at concentrations of ng/ml). Each protein was cleaved by cyanogen bromide and the resultant peptides, separated by HPLC, were sequenced as far as the Edman degradation allowed, giving a total of 61 amino acid residues for gp22 and 105 residues for gp35, which represent 30–50% of their expected length. Computer analyses of the sequenced segments revealed no significant homology to human β-interferon, each other, or any other recorded sequence.**

In many instances, plants react to virus infection by stimulating the production or activation of antiviral factors (designated in this laboratory as AVF; for a review, see ref. 1). In tobacco, the stimulation of AVF activity is associated with a single dominant gene (*N*) that confers a type of resistance on the plant by localizing tobacco mosaic virus (TMV) infection (2, 3). Hitherto, AVF purification was quite unwieldy, resulting in several fractions exhibiting antiviral activity and very little protein. Though a protein similar to glycoprotein (gp) 22 has been purified, only 1–2 μg of protein were obtained, barely allowing analysis by electrophoresis and characterization of activity (4). At least some forms of AVF are phosphorylated glycoproteins (5). Some evidence suggests that a certain level of pre-AVF is present in every tobacco cultivar and that pre-AVF is processed to active AVF after infection, especially in *N* gene-carrying plants (6). Several common features were found between AVF and interferon (IFN; ref. 7). Experiments carried out in this and associated laboratories indicated that treating plant tissue with either AVF or IFN stimulated the activity of an enzyme that polymerizes ATP to antiviral oligoadenylates (8, 9). The possible activity of human IFNs in plants was investigated. Indeed, in several studies, human IFNs inhibited the multiplication of TMV and other plant viruses (10–14); however, some other laboratories failed to observe antiviral activity of human IFN in plants (15, 16). IFN-induced oligoadenylates as well as synthetic 2′–5′-oligoadenylates also inhibited TMV multiplication in plants (17).

In this study we report the purification to homogeneity and the partial sequencing of two virus-stimulated antiviral tobacco proteins (AVFs, gp22 and gp35). Immunoaffinity chromatography on an immobilized monoclonal antibody to human IFN-β was a key step in this purification. The analogy of these plant proteins to the human IFN system is discussed.

MATERIALS AND METHODS

Plants. The antiviral plant proteins were isolated from the tobacco (*Nicotiana tabacum*) cultivar "Samsun NN," carrying the *N* gene, which determines the localization of TMV infection.

Preparation of Crude Plant Proteins. Tobacco leaves were inoculated with TMV and, after 48 hr, leaves were homogenized in 10 mM sodium phosphate (pH 7.6) and treated with hydrated calcium phosphate, as described by Sela (4). This treatment removed about 85% of the proteins as well as TMV, leaving a noninfectious antiviral fraction. Prior to large-scale immunoaffinity purification, this fraction was dialyzed against water and freeze-dried. In Western blottings of the crude preparation, the calcium phosphate step was omitted.

Immunoaffinity Purification of Tobacco Proteins with Monoclonal Antibodies to Human IFN-β. Crude protein preparations were dissolved in isotonic phosphate-buffered saline (PBS) and loaded onto a column of immobilized monoclonal antibodies (clone 117-1, IgG1) to human IFN-β (10 mg of antibodies bound to 1 ml of agarose polyacrylhydrazide; ref. 18 and 19). After washing with 30 ml of PBS, the bound proteins were eluted (ten 1-ml fractions) with 50 mM citric acid (pH 2.0). To reduce the adsorption of the eluted protein to the walls of the test tubes, the fractions were collected into tubes containing 0.25 ml of propylene glycol.

Protein Determination. Protein concentration was determined either by the Bradford method (20) or by fluorescamine (21). In both cases crystalline bovine serum albumin was used as a standard.

HPLC Separation of Proteins. Peak fractions, containing the eluate from the immunoaffinity column, were applied to an Aquapore RP-300 cartridge column (4.6 × 30 mm; Brownlee Lab) in 0.3% trifluoroacetic acid. Samples were eluted by an acetonitrile gradient at a flow rate of 0.5 ml/min. The column was monitored by an automated fluorescamine system (21).

CNBr Cleavage. Protein fractions obtained by reversed-phase HPLC were dried in vacuum and redissolved in 70% (vol/vol) formic acid (100 μl) containing CNBr (5 mg/ml). The reaction mixtures were left overnight in the dark at room temperature. The solutions were then applied directly to the Aquapore RP-300 column and bound peptides were eluted by an acetonitrile gradient in 0.3% trifluoroacetic acid.

Protein Sequencing. Intact proteins and their CNBr fragments, which were resolved by reversed-phase HPLC, were sequenced by using the microsequencing system that con-

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Abbreviations: TMV, tobacco mosaic virus; AVF, antiviral factor; IFN, interferon; PR, pathogenesis related.

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sisted of a pulse-liquid gas-phase microsequencer (model 475), an on-line phenylthiohydantoin HPLC analyzer (model 120), and an on-line data processing unit (model 900; all from Applied Biosystems).

Homology Search. Protein sequences were compared for homology by using the FASTP program (22) and the National Biomedical Research Foundation protein sequence data base (release no. 16).

Electrophoresis and Immunoblotting. Polyacrylamide gel electrophoresis of proteins was carried out in 7.5%–12.5% polyacrylamide gels, in the presence of SDS, according to Laemmli (23). The gels were stained by either the silver stain method (24) or by the Schiff reagent for glycoproteins (25). The glycoprotein nature of the immunopurified proteins reported herein was also determined by reacting their blots with peroxidase-coupled concanavalin A (26).

Electroblotting onto nitrocellulose membranes was performed essentially according to Towbin *et al.* (27). The nitrocellulose filters, incubated at 37°C for 1 hr in PBS containing 0.5% Tween 20 (Sigma) and 5% (wt/vol) low-fat-powdered milk, were transferred to the same solution containing rabbit polyclonal antibodies to human IFN-β (made by repeated immunization with homogeneous natural IFN-β; specific activity 3×10^8 units/mg), and incubated for 2 hr. Excess antibodies were removed by five washing periods in PBS/Tween. The incubation was continued for another 90 min after adding 10^6 dpm of ^{125}I-labeled protein A. The filters were then washed 10 times with PBS/Tween and subjected to autoradiography.

Assays for Antiviral Activity. Antiviral activity in plants was assayed by the leaf-disc test (4). Discs, punched out of tobacco leaves ("Samsun nn," allowing the systemic spread of TMV) and inoculated with the virus, were distributed at random in Petri dishes containing various dilutions of the tested proteins in 10 mM sodium phosphate (pH 7.6). After 72 hr of incubation at 28°C, TMV content was determined by ELISA (28).

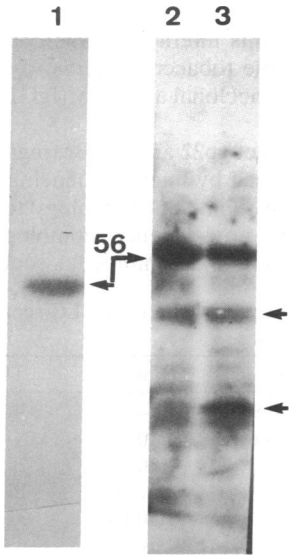

FIG. 1. Western blot analysis of crude tobacco proteins. Protein (30 μg per lane) was electrophoresed, electroblotted onto nitrocellulose, and incubated with polyclonal antibodies (dilution 1:500) to human IFN-β (lanes 2 and 3) or to collagen (lane 1). Lanes 1 and 2 carry protein extracts from noninfected tobacco and lane 3 carries a protein extract from TMV-infected tobacco (48 hr after inoculation). Arrows on left indicate the positions of gp22 and gp35. Nonspecific reaction to a 56-kDa protein is indicated by the arrow in the middle. Human IFN-β was immunopurified (specific activity, $>10^8$ units/mg) prior to rabbit immunization.

Table 1. Yield of IFN-β-analogous tobacco proteins after immunoaffinity purification

Source	Crude protein applied, mg	Protein bound, μg	Bound protein/ total protein
Noninfected leaves	127	56	0.04
TMV-infected leaves	124	155	0.12

Fraction applied to the immunoaffinity column was extracted from 230 g of leaves as indicated.

RESULTS

Western blot analyses of crude tobacco proteins (from healthy and TMV-infected plants) were performed with polyclonal antibodies to human IFN-β, as a part of a search for IFN-related proteins in virus-infected plants. Two major proteins (size estimate: 22 and 35 kDa, ±10%), later found to be TMV-stimulated, reacted with the antibodies (Fig. 1, lanes 2 and 3). At this stage, however, in which a crude extract of tobacco proteins was analyzed, some minor bands were observed as well. A number of control experiments were carried out to ascertain the specificity of this protein–antibody reaction. Western blot analyses of the crude tobacco protein extracts were performed with the following components: various batches of sera from IFN-β-immunized and nonimmunized rabbits, a rabbit-produced antiserum against collagen or several other plant and virus proteins [pathogenesis-related (PR) proteins, ribulose bisphosphate carboxylase/oxygenase, and TMV coat protein]. A specific reaction with immunogen was obtained in every blot. However, with some antisera, a nonspecific band, corresponding to a protein of molecular mass 56 kDa, was observed (Fig. 1, lane 1).

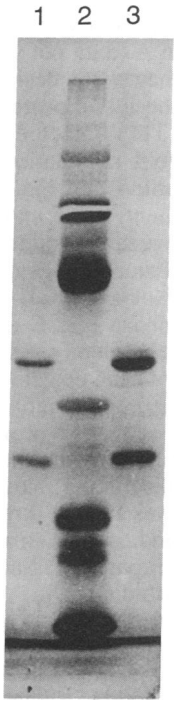

FIG. 2. Stimulation of gp22 and gp35 by TMV infection. Crude protein, 127 mg from noninfected tobacco leaves and 124 mg from TMV-infected leaves, 48 hr after inoculation, was applied to the monoclonal anti-IFN-β immunoaffinity column. The citric acid eluate was then electrophoresed and silver-stained. Lanes: 1, eluate from noninfected leaves; 2, size markers; 3, eluate from TMV-infected leaves.

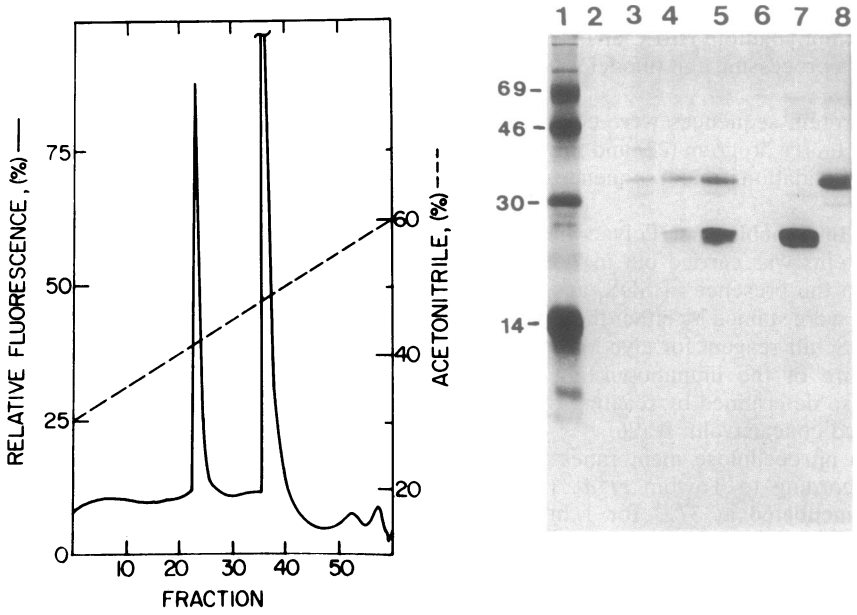

FIG. 3. Reversed-phase HPLC separation of gp22 and gp35. The citric acid eluate of the IFN-β immunoaffinity column was applied to the HPLC column. (*Left*) Elution profile of the HPLC column. (*Right*) Electropherogram of various fractions. Lanes: 1, molecular weight markers; 2–5, immunoaffinity column eluate fractions subjected to HPLC separation; 6, empty; 7, HPLC fraction 24 (gp22); 8, HPLC fraction 38 (gp35).

The plant proteins that reacted specifically with polyclonal antibodies to human IFN-β were then isolated by immunoaffinity chromatography. Clarified tobacco homogenates were applied to the immunoaffinity column, carrying monoclonal anti-human IFN-β antibodies. After extensive washings the bound proteins were eluted at low pH. Electrophoretic analysis of the eluted fractions revealed two major polypeptides, corresponding to the two proteins in the crude tobacco extracts that had reacted with anti-IFN-β polyclonal antibodies. Since both proteins reacted with the Schiff reagent and concanavalin A (data not shown), indicating their glycoprotein nature, they were designated gp22 and gp35. About 100–200 μg of these two proteins were thus obtained from 300 g of fresh TMV-infected tobacco leaves. This represents a 3- to 5-fold stimulation over the yield from noninfected leaves (Table 1 and Fig. 2).

In a control experiment, no bound protein was detected when a tobacco homogenate applied to an immunoaffinity column of immobilized monoclonal antibodies to dinitrophenol that had the same isotype (IgG1) as the IFN monoclonal antibodies. Reversed-phase HPLC of eluates from the immunoaffinity column completely resolved gp22 from gp35 (Fig. 3). About 100 μg of each protein was obtained, enabling further studies of structure and activity.

The antiviral activity of the two proteins was determined in leaf discs. Both proteins were found to be highly active in inhibiting TMV multiplication (Tables 2 and 3). The recovery of biological activity was two to three orders of magnitude higher than expected, indicating a strong antagonistic activity to AVF in the crude preparation. Such antagonistic activity has already been reported (1, 29).

N-terminal sequence analysis of gp22 gave a single sequence of 32 amino acid residues (see Fig. 5). This single sequence indicates that gp22 is a homogeneous protein containing a single polypeptide. Sequencing the intact gp-35 yielded no phenylthiohydantoin amino acid derivatives, indicating that its N terminus is blocked.

Proteins gp22 and gp35 were cleaved with CNBr and the CNBr peptides were separated by reversed-phase HPLC (Fig. 4). The sequence of each resolved peptide was then analyzed. In all, 61 amino acid residues of gp22 and 105 of gp35 were determined (Fig. 5).

The sequences of gp22 and gp35 were compared to each other and to those of human IFN-β, TMV-induced PR proteins (PR-1b and the PR basic chitinase), and every protein listed in the NBRF protein library. No significant homology was found.

DISCUSSION

Two antiviral proteins, gp22 and gp35, were purified to homogeneity from TMV-infected tobacco leaves by immunoaffinity chromatography on immobilized monoclonal antibodies to human IFN-β and then by reversed-phase HPLC. The specificity of this interaction was corroborated by the fact that none of the tobacco proteins was retained by the same isotype of monoclonal antibody (IgG1) directed against dinitrophenol.

Sequences of intact gp22 and CNBr fragments of gp22 and gp35 were determined by microsequencing, and a computer search revealed no significant homology to any known protein. Surprisingly, no sequence homology was found to human IFN-β either. The homologous sequences may lie in

Table 2. Antiviral activity data for HPLC-separated IFN-analogous proteins

Fraction	Dilution	TMV yield
HPLC buffer	—	30,900
Crude extract	1:10	<8 (100)
	1:100	25,700 (17)
	1:1,000	15,800 (49)
	1:10,000	31,800 (0)
22-kDa peak	1:10	<8 (100)
	1:100	<8 (100)
	1:1,000	8,900 (81)
	1:10,000	21,800 (30)
35-kDa peak	1:10	<8 (100)
	1:100	—
	1:1,000	<8 (100)
	1:10,000	7,900 (74)

TMV yield is expressed as ng of TMV per 25 discs treated with the respective fraction. Numbers in parentheses are percentage inhibition of TMV multiplication.

Table 3. Purification for HPLC-separated IFN-analogous proteins

Fraction	Protein, mg	Specific activity, units/mg of protein	Purification, fold
Crude	12.4	80	—
22-kDa peak	0.06	1×10^6	13,125
35-kDa peak	0.094	$>2.1 \times 10^6$	>26,625

Specific activity is stimulated protein concentration inhibiting TMV multiplication by 50%.

the still unsequenced portion of these proteins. Alternatively, the immunoreactive epitopes may be formed by folding, thus bringing amino acids from various places in the primary sequence into close proximity, a homology that could not be detected by analysis of the linear sequences. Such a situation has been observed for the two intensely sweet proteins, monellin and thaumatin that bear no structural similarity yet their antibodies cross-react (30).

The stimulation of PR proteins in TMV-infected tobacco plants was studied. Their role in the plant defense mechanism (if any) as well as in the hypersensitive reaction of localized TMV infections in tobacco is still debatable (31, 32). It is, therefore, noteworthy that gp22 and gp35 bear no structural resemblance to known PR-protein sequences, as indicated by the sequence analyses and the lack of cross-reactivity between gp22 or gp35 and antibodies to various PR proteins.

AVF, an antiviral factor stimulated in many plant species by a variety of viruses (1), is particularly active in TMV-infected *N* gene-carrying tobacco cultivars (2, 3). The basal level of AVF, seemingly present in all plants, is increased, activated, or both by virus infection to produce active AVF (6). A laborious procedure of purification yielded a minute amount of a 22-kDa protein, considered to be a species of AVF (4). By definition, gp22 and gp35 are AVF species. It is likely, but not certain, that the previously isolated 22-kDa protein (4) and gp22 are identical. The "IFN-like" proteins reported in this study resemble AVF in glycosylation, antiviral activity, occurrence at a basal level in tobacco plants, and induction by TMV infection.

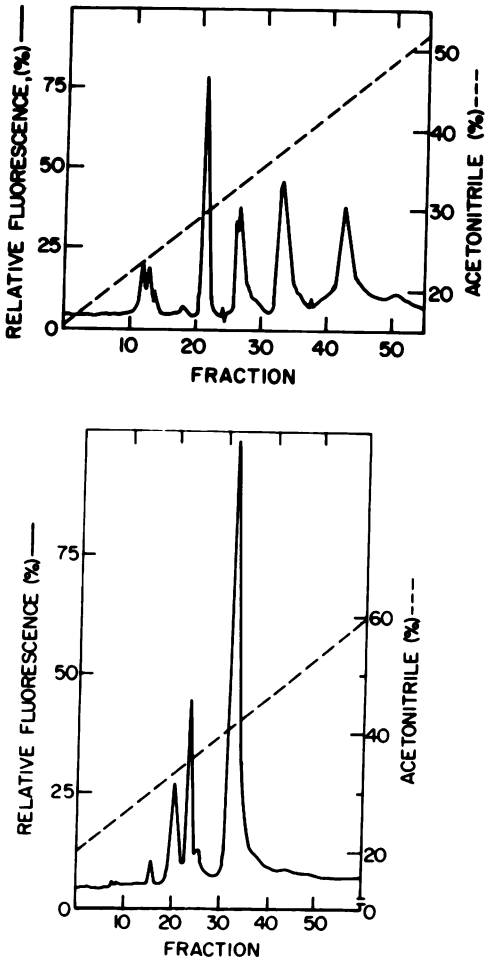

FIG. 4. Separation profiles of the peptides generated by CNBr cleavage of gp22 (*Lower*) and gp35 (*Upper*).

gp-22, HPLC fraction 16[1]

MET[2]-SER-ARG-GLU-GLY-HIS-ARG-TRP-HIS-PRO-TRP...

gp-22, HPLC fraction 20

MET-SER-PHE-GLY-PRO-THR-LYS-PRO-GLY-PRO-GLY-LYS-TYR-HIS-VAL-ILE-GLN-ALA...

gp-22, HPLC fraction 24 (N-terminus)

SER-GLY-VAL-PHE-GLU-VAL-HIS-ASN-ASN-ALA-PRO-TYR-THR-VAL-TRP-ALA-ALA-ALA-THR-PRO-VAL-GLY-GLY-GLY-LYS-ARG-LEU-GLU-ARG-SER-GLN-SER...

gp-35, HPLC fraction 21

MET-PHE-ASP-GLU-ASN-ASN-LYS-ASN-PRO-GLU-LEU-GLU-LYS-HIS-PHE-GLY-LEU-PHE-SER-PRO-ASN-LYS-GLN-PRO-LYS-TYR-ASN-LEU-ASN-PHE-GLY...

gp-35, HPLC fraction 27

MET-LEU-GLY-ASN-ASN-LEU-PRO-ASN-HIS-?-GLU-VAL-ILE-GLN-LEU-TYR-LYS-SER-ARG-ASN-ILE-GLY-GLY-LEU...

gp-35, HPLC fraction 33

MET-GLU-HIS-ALA-ARG-TRP-TRP-VAL-GLN-LYS-ASN-VAL-LYS-ASP-PHE-TRP-PRO-ASP-VAL-LYS-ILE-LYS-TYR-ILE-ALA-VAL-GLY-ASN-GLU...

gp-35, HPLC fraction 43

MET-VAL-ASN-ILE-TYR-LYS-ALA-ILE-PRO-GLU-ALA-GLY-LEU-GLY-ASN-ASN-ILE-LYS-VAL-SER-THR-SER-VAL-ASP-THR...

FIG. 5. Amino acid sequences of the CNBr-generated fragments of gp22 and gp35. [1]See Fig. 4. [2]Methionine was assumed to be the last amino acid on any preceding fragment except for the N terminus.

The resemblance of AVF to IFN has been discussed (7) and antiviral activity of human IFNs in plants has also been reported (10–14). The AVFs isolated in this study presumably fold to create sites recognized by antibodies to human IFN-β and this may also explain the antiviral activity of human IFN in plants. In this respect it is noteworthy that monellin and thaumatin also share a common biological activity.

Our data support, in a more definitive manner, previously reported (7, 9, 10, 17) evidence for the presence of IFN-analogous systems in plants.

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