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DETECTION OF TOMATINASE FROM *FUSARIUM OXYSPORUM* F. SP. *LYCOPERSICI* IN INFECTED TOMATO PLANTS

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Key Word Index—*Fusarium oxysporum* f. sp. *lycopersici*; *Lycopersicon esculentum*; tomatinase; α-tomatine; saponins.

Abstract—The antifungal glycoalkaloid α-tomatine of the tomato plant (*Lycopersicon esculentum*) is proposed to protect the plant against phytopathogenic fungi. *Fusarium oxysporum* f. sp. *lycopersici*, a vascular pathogen of tomato, produces a tomatinase enzyme which hydrolyses the glycoalkaloid into non-fungitoxic compounds. Detoxification of α-tomatine may be how this fungus avoids the plant glycoalkaloid barrier. As an initial step to evaluate this possibility we have studied the induction of tomatinase; (i) in fungal cultures containing extracts from leaf, stem or root of tomato plants; and (ii) in stem and root of tomato plants infected with the pathogen at different infection stages. The kinetics of tomatinase induction with leaf extract (0.6% dry weight) was similar to that observed with 20 $\mu g\ ml^{-1}$ of α-tomatine. In the presence of stem extract, tomatinase activity was less than 50% of that induced with leaf extract, whereas in the presence of root extract tomatinase activity was very low. In the stem of infected tomato plants tomatinase activity was higher at the wilt stage than in previous infections stages and in root, tomatinase activity appeared with the first symptoms and was maintained until wilting. TLC analysis showed that the tomatinase induced in culture medium with plant extracts and in infected tomato plants had the same mode of action as the enzyme induced with pure α-tomatine, hydrolysing the glycoalkaloid into its non-fungitoxic forms, tomatidine and β-lycotetraose. The antisera raised against purified tomatinase recognized in extracts of root and stem of infected tomato plants a protein of 50 000 (45 000 when proteins were deglycosylated), corresponding to the tomatinase enzyme. Therefore, it is concluded that *F. oxysporum* f. sp. *lycopersici* express tomatinase *in vivo* as a result of the infection of tomato plant.

INTRODUCTION

Plants have evolved different mechanisms to protect themselves against a great variety of invasive pathogens. Antifungal saponins are produced by many plants, including many major food crops [1], and they have been implicated as preformed determinants of resistance to fungal attack [2–5]. Saponins consist of a triterpenoid, steroid or steroidal alkaloid bearing one or more sugar chains [1, 6]. Recently, the term phytoanticipin has been proposed to describe saponins and other low molecular weight antimicrobial compounds that are produced as part of normal plant development and that might function to protect plants from disease [7]. The saponin α-tomatine is a steroidal glycoalkaloid found in the leaf, stem, root, flower and green fruit of the tomato plant (*Lycopersicon esculentum*). It consists of an aglycone moiety (tomatidine) and a tetrasaccharide moiety (β-lycotetraose) which is composed of two molecules of glucose and one each of galactose and xylose [8]. Because of its antifungal property, α-tomatine has been suggested to protect *Lycopersicon* species from general microbial infection [5, 8, 9, 11, 12]. The presumed fungitoxic effects of α-tomatine and other saponins are attributed to their interaction with 3β-hydroxy sterols, causing an increase of membrane permeability, pore formation and leakage of cells contents [13–18].

Previous studies have shown that, in general, tomato pathogens are less sensitive to α-tomatine than are most non-pathogenic fungi [9, 18]. Some fungi are resistant to the toxic effects of α-tomatine because they have little or no sterols in their membranes composition [9, 19], while others produce specific α-tomatine-detoxifying enzymes [5, 10, 11, 20–23]. These enzymes, known as tomatinases, are, in most cases, inducible by α-tomatine but differ in their properties and in their mode of detoxification of α-tomatine [10, 11, 20–23]. *Fusarium oxysporum* is a widespread soil-borne plant pathogen causing general vascular wilts. It exists as many forms grouped into formae speciales

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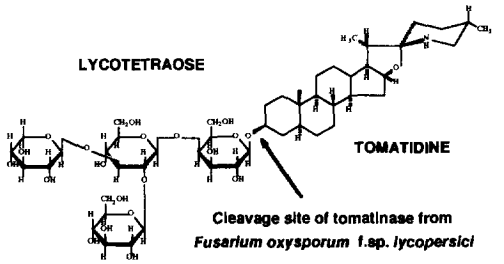

Fig. 1. α-Tomatine structure and site of cleavage by tomatinase from *F. oxysporum* f. sp. *lycopersici*.

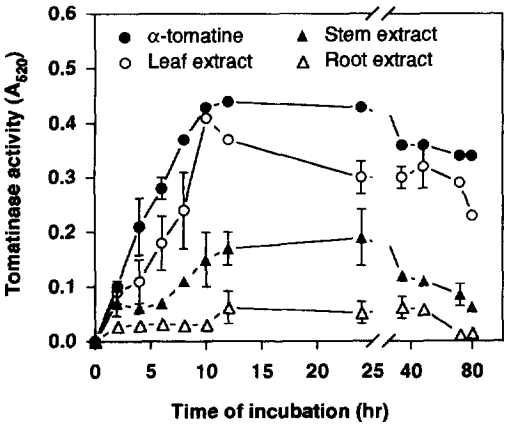

Fig. 2. Kinetics of tomatinase induction in *Fusarium oxysporum* f. sp. *lycopersici* 4287 (*Fol*) grown in the presence of tomato plant extracts: *Fol* grown in the presence of 20 μg ml^{-1} of pure α-tomatine (●) tomato leaf extract (0.6%) (○), tomato stem extract (0.6%) (▲) and tomato root extract (0.6%) (△). Tomatinase activity is expressed as A_{520} of mixtures consisting of 0.1 ml of crude extract in a final volume of 0.2 ml (20 mM sodium acetate pH 5.5 and 1 mM tomatine) after 30 min incubation at 37°. Each point represents the mean of three dependent experiments and vertical bars the standard error.

on the basis of their ability to invoke disease in a particular host [24]. *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) (Sacc) Snyd. & Hans. causes vascular wilt of tomato, the cause of severe crop losses throughout the world [25, 26]. Tomatinase from *Fol*, inducible by α-tomatine, removes all four sugars by cleaving the β,1-linked galactose and releasing the tetrasaccharide lycotetraose and tomatidine [10, 21] (Fig. 1). Such a mode of action is similar to that reported for the tomatinase from *Botrytis cinerea* [12]. The tomato pathogen *Alternaria solani* also degrades α-tomatine to tomatidine, but does so by a release of monosaccharides rather than a tetrasaccharide [5]. Tomatinase from *Septoria lycopersici*, as tomatinase from *Verticillium albo-atrum*, removes a single sugar, the terminal β,1-2-linked glucose from α-tomatine [11, 20]. In all cases the deglycosylation may be sufficient to destroy the ability of the α-tomatine to complex with membrane sterols, and therefore eliminates its toxic effect [27]. Therefore, the detoxification of α-tomatine may be how these fungi avoid the tomato glycoalkaloid barrier.

As an initial step to evaluate this possibility, we have studied the induction of tomatinase from *Fol* (i) in fungal cultures containing extracts from leaf, stem or root of tomato plants; and (ii) in stem and root of tomato plants infected with the pathogen at different infection stages. In both cases we were able to detect the enzyme, by means of a tomatinase activity assay, thin-layer chromatography (TLC) and Western blotting analysis. To date, α-tomatine detoxifying enzymes from different tomato pathogens have only been detected *in vitro*; here we report for the first time the expression of tomatinase from *Fol* inside the infected tomato plant.

RESULTS AND DISCUSSION

Tomatinase induction in the presence of tomato plant extract

Figure 2 shows the induction kinetics of tomatinase activity from *Fol* in fungal culture containing 0.6% (dry weight) of leaf, stem or root extract. The kinetics of tomatinase induction in fungal culture containing leaf extract was comparable to that of *Fol* grown in the presence of 20 μg ml^{-1} α-tomatine. The tomatinase activity in filtrates from *Fol* grown in the presence of leaf extract was more than double that found in filtrates of the fungus grown in the presence of stem extract. In the presence of root extract, only very low activity was detected. No activity was detected in filtrates from *Fol* grown in absence of α-tomatine.

Research into the biological activity of α-tomatine was initiated by the finding that a crude extract of α-tomatine inhibited the *in vitro* growth of *Fol* [8]. In the present study, after 24 hr incubation, a higher mycelial dry weight was recorded for *Fol* when grown in the presence of tomato root extract (0.244 g ± 0.029) in comparison to that observed when the fungus was grown in the presence of tomato stem or leaf extract (0.202 g ± 0.016 and 0.188 g ± 0.015, respectively). The mycelial dry weight of *Fol* recorded in tomatine-free controls was very similar to that observed in the presence of root extract (0.25 g ± 0.011). These results are highly correlated with the α-tomatine content of each organ of the tomato plant. Hence, as reported by several authors [8, 28], α-tomatine was found at higher concentrations in leaves (0.46–5.1%) compared to stem and root (0.08–0.6%). These differences in α-tomatine levels and its toxic effect explain the high tomatinase induction (Fig. 2) and the high mycelial growth inhibition observed when *Fol* was grown in the presence of a leaf extract.

Tomatinase induction in the infected tomato plant

Tomatinase activity in infected tomato plants was recorded at different stages of infection in the stem and root (Table 1). In the leaf, no tomatinase activity was detected at any stage of infection (data not shown). In the stem, tomatinase activity was higher at

Table 1. Tomatinase activity in the stem and root of a tomato plant infected by *F. oxysporum* f. sp. *lycopersici**

Tomato plant organ	Tomatinase activity (A_{520})† S_1‡	S_2	Wilt
Root	0.4	0.35	0.44
Stem	0.15	0.14	0.75

* Representative experiment.

† Tomatinase activity after incubation overnight at 37° of the protein extract with α-tomatine.

‡ Stage of infection of the tomato plant: S_1 (stem at stage of yellowing or wilting of the lower leaves), S_2 (stem at stage of yellowing or wilting of all the leaves) and Wilt (the entire plant showing wilting).

the wilt stage than at stages 1 and 2 of infection. In the root, enzyme activity appeared with the first symptoms (stage 1), and was maintained until wilting. Tomatinase activity was not detected in protein extracts from the stem and root of infected plants without apparent symptoms (1–5 days after infection). This fact may be due to the low presence of the pathogen in the plant and also to the inhibiting effect of α-tomatine on spore germination and mycelial growth. This also may explain the very low tomatinase activity observed in the stem of infected tomato plant at stages 1 and 2 of infection, stages at which the pathogen spores reach the tomato stem and proliferate in it. This activity was also absent in protein extracts from the stem and root of healthy plants, thus ruling out the possibility of the presence in the plant of enzymes with similar tomatinase activity.

Mode of action of tomatinase

TLC analysis showed that the tomatinase detected in all cases in extract-treated cultures, had the same mechanism of action as the tomatinase induced with pure α-tomatine. The enzyme degrades α-tomatine into two products, tomatidine and β-lycotetraose. Tomatidine was visible on the TLC plate as a fast-moving spot with respect to α-tomatine [Fig. 3(A)]. The TLC analysis confirmed the results presented in Table 1 where the tomatinase activity in the stem was higher at the wilt stage than at stages 1 and 2 of infection [Fig. 3(A)]. In the root, although only a low activity was detected, it was clearly observed from stage 1 to wilting [Fig. 3(A)]. No degradation of α-tomatine occurred when protein extracts from the root and stem of infected plants without symptoms (1–5 days after infection) were tested [Fig. 3(B)]. Moreover, TLC confirmed the absence of tomatinase activity in protein extracts from the stem and root of healthy plants [Fig. 3(B)].

Western blotting analysis

An immunoblotting assay was set up using rabbit polyclonal antibodies raised against tomatinase from

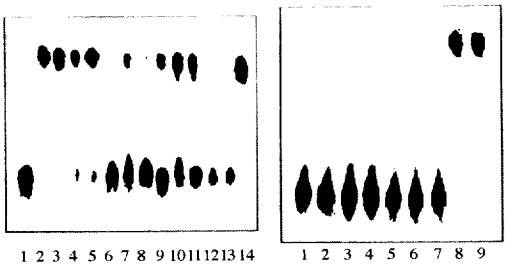

Fig. 3. Thin layer chromatography to tomatinase reaction products. Protein extracts were incubated overnight at 37° in the presence of 1 mM α-tomatine: (A) Lanes: **1**, α-tomatine; **2**, **3**, **4** and **5**, culture filtrates of *Fol* grown in the presence of α-tomatine (positive control), tomato leaf, stem and root extract respectively (10 μg of total protein each); **6** and **7**, infected tomato stem and root extract, respectively, at stage 1 of infection (50 μg of total protein each); **8** and **9**, infected tomato stem and root extract, respectively, at stage 2 of infection (50 μg of total protein each); **10** and **11**, infected tomato stem and root extract, respectively, at the wilt stage of infection (50 μg of total protein each); **12** and **13**, healthy tomato stem and root extract respectively (50 μg of total protein each) and **14**, tomatidine. (B) Lanes: **1**, α-tomatine; **2** and **3**, infected tomato stem and root extract, respectively, 5 days after infection (50 μg of total protein each); **4** and **5**, infected tomato stem and root extract, respectively, 24 hr after infection (50 μg of total protein each); **6** and **7**, healthy tomato stem and root extract, respectively (50 μg of total protein each); **8**, positive control (10 μg of total protein) and **9**, tomatidine.

Fol. The antisera showed the high presence of a 50 000 band in the crude protein preparation from a culture filtrate of *Fol* grown in the presence of leaf extract [Fig. 4(A)]. This band was assumed and later demonstrated to correspond to the tomatinase enzyme. The 50 000 band was slightly detected in protein extracts from culture filtrates of *Fol* grown in the

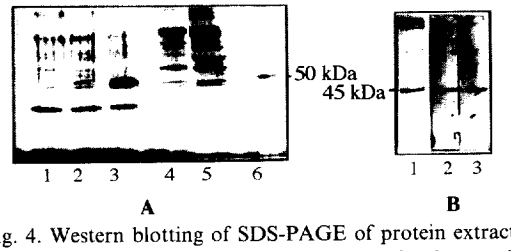

Fig. 4. Western blotting of SDS-PAGE of protein extracts from: (A) Lanes: **1**, **2** and **3**, culture filtrates of *Fol* grown in the presence of tomato root, stem and leaf extract respectively (8 μg of total protein each); **4** and **5**, infected tomato root and stem extract, respectively, at the wilt stage of infection (25 μg of total protein each); **6**, culture filtrate of *Fol* grown in the presence of α-tomatine, positive control showing the 50 000 band of tomatinase enzyme (5 μg of total protein). (B) Deglycosylated protein extracts from–Lanes: **1**, culture filtrate of *Fol* grown in the presence of α-tomatine, positive control showing the 45 000 band of deglycosylated tomatinase (10 μg of total protein); **2**, infected tomato stem extract at the wilt stage of infection (50 μg of total protein); **3**, culture filtrate of *Fol* grown in the presence of tomato stem extract (30 μg of total protein).

presence of stem and root extracts [Fig. 4(A)], due to the low levels of α-tomatine in these organs of tomato plant. This band was also detected in protein preparations from root and stem of plants at wilt stage of infection [Fig. 4(A)]. No bands were observed when protein extracts from a healthy plant were screened (data not shown) indicating that all the proteins detected by the antisera were of fungal origin. The nonspecific reactions of the antisera with proteins other than tomatinase were due to those antibodies recognizing carbohydrate moieties of tomatinase also present in other proteins. Previous work has shown that *N*-glycosidase F hydrolyses all the oligosaccharides moieties from tomatinase giving rise to a band of 45 000 that corresponds to the deglycosylated form of tomatinase [21]. Thus, to confirm the presence of tomatinase and resolve the problem of false positives, crude extracts from *Fol* grown in the presence of tomato stem extract and from infected tomato stem were treated with *N*-glycosidase F and then subjected for Western blotting. In this case only a band of 45 000, corresponding to the deglycosylated form of tomatinase was detected [Fig. 4(B)]. These results demonstrate the uniqueness of the tomatinase induced both *in vivo* and *in vitro*.

In order to confirm the above conclusions, native-polyacrylamide gel electrophoresis (10% acrylamide) was set up using protein extracts from culture filtrates of *Fol* grown in the presence of leaf, stem and root extracts, and from root and stem of a tomato plant at the wilt stage of infection. After running the protein preparations, each gel was cut into slices of 0.5 cm and tomatinase activity was determined in each slice, as previously described [21], followed by TLC analysis. The results obtained revealed that tomatinase activity was exclusively associated with the 50 kDa protein (data not shown), thus ruling out the possibility of the existence of other proteins with tomatinase activity and confirming the presence of only one induced tomatinase in the tomato plant, which was the same in all the samples tested.

Although several authors have reported that the glycoalkaloid α-tomatine is not present in high enough concentrations in the root or stem to play a major role in resistance to *Fol* [8, 9, 29], the fact that tomatinase is induced in the tomato plant after *Fol* infection, suggests a possible role of this enzyme in pathogenicity. This role may be demonstrated by means of genetic manipulations. At present we are using different strategies to clone the gene encoding tomatinase from *Fol*. The cloned gene should allow us to obtain tomatinase-minus mutants by targeted gene disruption and to investigate directly the role of this enzyme in pathogenicity.

EXPERIMENTAL

Tomatinase activity in vitro. Microconidia were germinated in 25 ml medium consisting of casamino acids (Difco) (10 g l^{-1}) ammonium sulphate (10 mM) and Yeast Nitrogen Base (Difco) (0.5 g l^{-1}) for 16 hr at 28°, collected by centrifugation (10 000 *g* for 10 min), resuspended in 100 ml fresh medium and incubated 24 hr at 28°. Roots, stem and leaf of tomato plants (4 weeks old) were lyophilized separately and 0.6 g (dry wt) of each, was pulverized under liquid N_2 in a mortar with pestle. The powder was recovered in 5 ml citric acid pH 4 and homogenized. The homogenate was centrifuged at 14 000 *g* for 20 min at 4°. The supernatant was filtered through a Millipore filter (0.22 μm) and added to the *Fol* mycelium. Positive control consisted of *Fol* incubated in the presence of 20 μg ml^{-1} of α-tomatine (Sigma) dissolved in 50 mM potassium citrate buffer (pH 4), and the negative control consisted of fungus incubated in α-tomatine free buffer. Tomatinase activity was determined at different times during incubations.

For Western blotting and TLC analysis, cultures at maximum tomatinase activity were filtered through nylon cloth, centrifuged at 10 000 *g* for 15 min, and the supernatant filtered through a Millipore filter (0.22 μm). The filtrates were concd × 10 with polyethylene glycol 35 000 [21] and proteins were ppd by addition of 2.5 vol. of cold Me_2CO (−80°) under slow agitation. Finally, the pellet obtained after centrifugation at 14 000 *g* for 25 min at 4°, was dissolved in sterile milli-Q H_2O and stored at 4° until use.

Tomatinase activity assay. Tomatinase activity was determined spectrophotometrically at 520 nm using the dinitrosalicylic acid reagent (DNS) for determination of reducing sugars [30, 31]. Protein extracts were incubated in the presence of α-tomatine for 30 min at 37° (except for protein extracts from infected plants where tomatinase activity was set up overnight at 37°). Negative controls consisted of each protein extract incubated in the presence of α-tomatine-free buffer. These controls, in the presence of DNS reagent, were used as blanks for the spectrophotometric determination of tomatinase activity. Other details of the activity estimation were as previously described [21].

Pathogenicity test. *Fol*, strain 4287, was grown in potato dextrose broth medium (Difco) for 5 days on an orbital shaker (120 rpm) at 28°. The culture was then filtered through nylon cloth to separate spores from mycelium. Seeds of tomato, cultivar Moneymaker, were surface sterilized in 1% sodium hypochlorite for 30 min and rinsed thoroughly with sterile distilled H_2O. Tomato seedlings were then grown in sterile conditions in a controlled environment (25°, 12 hr photoperiod). After 2 weeks (two-leaf stage) the root of tomato seedlings were dipped into the spore suspension (5×10^6 spores ml^{-1}) for 30 min and then transplanted to plastic cell trays containing vermiculite with one seedling per cell. Other seedlings were dipped in H_2O as controls. The susceptibility of tomato plants to *Fol* was recorded within 3 weeks. The first plant samples were collected 24 hr after inoculation, a second group of plants was collected 5 days after inoculation and identified as plants with no

apparent symptoms. Symptom expression emerged 7 days after inoculation; tomato plant wilt began on the 10th day. The rating of symptom expression was as follows: 0 = no symptoms; 1 = yellowing or wilting of the lower leaves; 2 = yellowing or wilting of all the leaves; w = the entire plant showing wilting. Samples of stems and roots of the collected plant were stored at −80° until use.

Protein extraction. Frozen stems and roots at different stages of infection (1.5 g each) were pulverized to a fine powder under liquid N_2 in a mortar with a pestle. The powder was collected in an appropriate vol. of extraction buffer (50 mM NaOAc, pH 5.5, 50 mM KCl, 5% glycerol, 2% borate, 0.1% Tween 80 and 5 mM EDTA). The homogenate was centrifuged at 14000 *g* for 20 min at 4° and proteins were ppd from the supernatant by addition, under low agitation, of 2.5 vol. of cold (−80°) Me_2CO followed by centrifugation at 14000 *g* for 30 min at 4°. The protein pellet was then resuspended in sterile milli-Q H_2O and stored at 4° until use.

TLC analysis. Protein extracts from (i) culture filtrates of *Fol* grown in the presence of α-tomatine, leaf, stem or root extracts, and from (ii) stem and root of infected and healthy tomato plants, were incubated overnight at 37° in the presence of α-tomatine (1 mM) in a final vol. of 200 μl (20 mM NaOAc buffer pH 5.5). After incubation, α-tomatine and tomatidine were ppd by increasing the pH to 10 with NaOH. The pellet (α-tomatine and/or tomatidine) was resuspended into 20 μl MeOH and sepd on silica gel 25 TLC developed with HoAc–EtOAc–MeOH–H_2O (10:30:20:1). Spots were visualized after being sprayed with 50% H_2SO_4 and heated at 110°. The metabolites were identified by co-chromatographic analysis with standards (α-tomatine and tomatidine both 1 mM). α-Tomatine appeared as a black spot near the origin, while tomatidine was localized as a green spot near the solvent front.

Antisera processing. Rabbit polyclonal antibodies were raised against the induced tomatinase from *Fol.* Crude extracts from culture filtrates of *Fol* grown in the presence of α-tomatine were concd with polyethylene glycol 35000 and dialysed overnight at 4°. Tomatinase was partially purified using prep. isoelectrofocusing, as previously described [21], and then sepd from other proteins by SDS-PAGE (11%). The band corresponding to tomatinase was cut from the gel, pulverized to a fine powder under liquid N_2 in a mortar with a pestle and recovered in phosphate-buffer saline (PBS) pH 7.4. Tomatinase suspension was then coupled to Freund's adjuvant and used as the immunogen. Tomatinase (around 100 μg) mixed with Freund's complete adjuvant (1:1) was given to the rabbit via multiple subcutaneous injections on the back. The animal was boosted four times more, at regular intervals of 15 days, with tomatinase (around 100 μg) coupled to Freund's incomplete adjuvant (1:1). Blood (30 ml) was drawn 7 days after each booster injection and the serum tested for the presence of anti-tomatinase antibody. The serum was stored at −20° until use. The antisera were cross-adsorbed by repeated incubations with the crude filtrate from *Fol* grown in the absence of α-tomatine. The IgGs were purified using Hitrap protein A (Pharmacia, Biotech.) according to the protocol of the manufacturer and quantified by measuring the absorbance at 280 nm.

Western blotting. Protein extracts were sepd by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 11% polyacrylamide gels [32]. Electrophoresis was performed as previously described [21]. After SDS-PAGE, proteins were transferred electrophoretically to nitrocellulose papers 0.45 μm pore size (Sigma) [33]. Protein transfer was set up overnight at 30 V (40–50 mA) in transfer buffer of 48 mM Tris, 39 mM glycine, 1.3 mM SDS and 20% MeOH. Nitrocellulose filters were incubated for 1 hr at room temp. in a blocking soln of Tris-buffer saline (TBS), pH 7.4, containing 2% non-fat dry milk, with gentle agitation. The primary antibody raised against tomatinase was then added to nitrocellulose membrane in blocking soln at a final conc. of 1:200 and incubated for 2 hr at room temp. After washing the filter ×5 (5 min each time) with TBS, pH 7.4, the nitrocellulose paper was incubated for 2 hr at room temp. with horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-rabbit IgG (Sigma Immunochemicals) (1:10000 in blocking solution). Binding of the secondary antibody was detected by the reaction of the antibody-HRP-conjugate with freshly prepd substrate soln consisting of 30 μl of 30% hydrogen peroxide, 10 ml of 0.3% 4-chloro-1-naphthol in methanol, and 50 ml of TBS for 10 to 20 min [34].

Protein deglycosylation. Protein extracts were deglycosylated using *N*-glycosidase F (Boehringer Mannheim, Germany). Deglycosylation was carried out following the instructions of the manufacturer.

Protein determination. Protein concn was determined as described by Bradford [35] using bovine serum albumin as standard.

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