High Affinity Binding of a Glycopeptide Elicitor to Tomato Cells and Microsomal Membranes and Displacement by Specific Glycan Suppressors*

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We have previously isolated glycopeptides derived from yeast invertase that acted as highly potent elicitors in suspension-cultured tomato cells, inducing ethylene biosynthesis and phenylalanine ammonia-lyase activity, and we have found that the high mannose oligosaccharides released from the pure glycopeptide elicitors by endo-β-N-acetylglucosaminidase H acted as suppressors of elicitor activity (Basse, C. W., Rock, K., and Boller, T. (1992) J. Biol. Chem. 267, 10258–10265). One of the elicitor-active glycopeptides (gp 8c) was labeled with t-butoxycarbonyl-[35S]methylmethionine and purified by reversed phase high performance liquid chromatography resulting in a specific radioactivity of the derivative of about 900 Ci/mmol. This radiolabeled glycopeptide showed specific, saturable, and reversible binding to whole tomato cells under conditions in which cells are responsive to elicitors as well as to microsomal membranes derived from these cells. Ligand saturation experiments, performed with microsomal membranes, gave a dissociation constant ($K_d$) of 3.3 nM as determined by Scatchard analysis. Various glycopeptide elicitors and preparations from yeast invertase were compared with respect to their abilities to compete for binding of $^{35}$S-labeled gp 8c to tomato membranes and to induce ethylene biosynthesis in tomato cells. These studies revealed a high degree of correlation between elicitor activities in vitro and displacement activities in vitro. In both tests, a high activity depended on the presence of glycans side chains consisting of more than 8 mannosyl residues. The high mannose oligosaccharides that acted as suppressors of elicitor activity in vitro competed for binding of the labeled elicitor as well. The suppressor-active glycan Man<sub>1</sub>GlCNac and the elicitor-active gp 8c exhibited very similar displacement activities, and the inhibitory constant ($K_i$) of the glycan Man<sub>1</sub>GlCNac was very similar to the $K_d$ value calculated for $^{35}$S-labeled gp 8c, indicating that the glycopeptide elicitors and the glycan suppressors derived from these elicitors competed with similar affinities for the same binding site. The suppressor-inactive glycan Man<sub>1</sub>GlCNac had a 200-fold lower capacity to compete for binding of $^{35}$S-labeled gp 8c to tomato membranes compared with the suppressor-active glycan Man<sub>1</sub>GlCNac. Our results demonstrate the existence of a specific elicitor binding site in tomato cell membranes and suggest that glycopeptides and glycans act as agonists and antagonists for induction of the stress response, respectively, by competing for this binding site.

Plants have a capacity to perceive specific components of microorganisms, so-called elicitors (Scheel and Parker, 1990), and to respond to elicitors by induction of physical and chemical defense responses such as phytoalexin biosynthesis, lignification (Dixon and Lamb, 1990), or a rapid formation of the plant hormone ethylene (Boller, 1990, 1991). Proteins, glycoproteins, peptides, oligosaccharides, and lipids are among the various classes of elicitors, which have mainly been isolated from fungi (Elbel and Scheel, 1992) but recently also from bacteria (Wei et al., 1992). We isolated highly potent glycopeptide elicitors and suppressors of a defense response in tomato cells (Lycopersicon esculentum (L.) Mill), using crude yeast extract (Basse and Boller, 1992) or yeast invertase digested by α-chymotrypsin (Basse et al., 1992) as starting materials. The most active of the invertase-derived glycopeptide elicitors induced ethylene biosynthesis and phenylalanine ammonia-lyase, the first enzyme in the phenyl propanoid pathway, half-maximally at a concentration of 5–10 nM. Further analysis indicated that the elicitor activity depended on both, the oligosaccharide moiety and the peptide moiety, and that the presence of a third α-1,6-linked mannose residue, present in oligosaccharides with more than eight mannosyl residues, was decisive for elicitor activity. The highest elicitor activities were obtained with glycopeptides containing oligosaccharides with 10–12 mannosyl residues. In contrast, there was no specific sequence requirement of the peptide moiety for elicitor activity (Basse et al., 1992). The peptide moieties of the elicitor-active glycopeptides contained only a few amino acids: gp 8, the most active glycopeptide, had the sequence Asn-Gly-Thr-His-Phe (Basse et al., 1992). Cleavage of glycopeptide elicitors by Endo H or N-glycanase completely inactivated elicitor activity and generated molecules that acted as suppressors of the induction by glycopeptide elicitors of ethylene biosynthesis and phenylalanine ammonia-lyase activity (Basse and Boller, 1992; Basse et al., 1992). Only oligosaccharides with more than 8 mannosyl residues acted as efficient suppressors, and thus the structural requirements for the free oligosaccharides to act as suppressors were the same as for the oligosaccharide side chains of the glycopeptides to give elicitor activity. This suggests that the glycan

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1 The abbreviations used are: Endo H, endo-β-N-acetylglucosaminidase H; ACC, 1-aminoacyclopropane-1-carboxylic acid; HPLC, high performance liquid chromatography; Boc-Met-OSu, t-butoxycarbonyl-L-methionine N-hydroxysuccinimidyl ester; $^{35}$S-labeled gp 8c, Boc-$^{35}$S-methionine sulfoxide-gp 8c; MeSO, dimethyl sulfoxide; MES, 2-(N-morpholino)ethanesulfonic acid.
suppressor occupies the same recognition site as the glycopeptide elicitor (Basse et al., 1992).

Indirect evidence for the presence of elicitor receptors has been obtained from various binding studies of radioactively labeled elicitor preparations to either microsomal fractions, plasma membrane preparations, or protoplasts (Dixon and Lamb, 1990). A first convincing report on specific binding of a β-glucan elicitor derived from Phytophthora megasperma was provided by Schmidt and Ebel (1987). A glucan fraction with phytoalexin-inducing activity and an average degree of polymerization of 22 was titrated to yield a specific radioactivity of 2 Ci/mmol. Scatchard analysis showed the binding site to be on the plasma membrane (Schmidt and Ebel, 1987). The specific radioactivity of the glucan fraction was increased by derivatization with tyramine, followed by labeling with 125I (Cosio et al., 1988). With the 125I-labeled glucan, binding in vivo to soybean protoplasts was shown. The highest binding affinity to a microsomal fraction from soybean roots was obtained with the 125I-labeled synthetic heptaglucoside (Cosio et al., 1990). The derivatized heptaglucoside gave a Kd of 3 nM as determined by Scatchard analysis. Various glucans differing in their degree of polymerization (Cosio et al., 1990) and several structural isomers of glucans ranging in size from hexamer to decamer (Cheong and Hahn, 1991) were used to compare their abilities to compete for binding of the 125I-labeled synthetic heptaglucoside to soybean membranes with their activities to induce phytoalexin accumulation in soybean cotyledons. These studies revealed a high degree of correlation between elicitor and displacement activities. Affinity labeling of β-glucan-binding proteins partially purified from soybean root membranes, with a 125I-labeled photolabile derivative of the heptaglucoside elicitor indicated a 70-kDa protein as the principal specific binding site of the hepta-β-glucoside (Cosio et al., 1992). Taken together, the binding site characterized by Cosio et al. (1990, 1992) and Cheong and Hahn (1991) has many of the characteristics expected for an elicitor receptor, although definitive proof is lacking.

In the present study, we have conjugated an elicitor-active glycopeptide from yeast invertase with [35S]methionine to demonstrate specific and high affinity binding to whole cells and to microsomal membranes from suspension-cultured tomato cells. We show that binding activity correlates with elicitor activity and that glycan suppressors and glycopeptide elicitors compete with very similar displacement activities for the putative receptor site.

**EXPERIMENTAL PROCEDURES**

**Plant Cells**—Tomato cells (line Msk 8) were grown as described (Felix et al., 1991) and used 9-14 days after transfer into fresh growth medium for assays of elicitor and suppressor activity (Bass and Boer, 1992). ACC synthase activity in tomato cells was measured as described by Spanu et al. (1990).

**Preparation of Microsomal Membranes from Tomato Cells**—Microsomes were prepared according to a procedure modified from Gal-lager and Leonard (1982) described by Grosskopf et al. (1990). Suspension-cultured tomato cells (7-10 days after transfer into fresh growth medium), were harvested on a sieve and the fresh weight determined. The cells were suspended in an equal volume of ice-cold homogenization buffer (50 mM Tris-MES, 3 mM MgCl2, 5 mM dithiothreitol, pH 7.5). After homogenization with a Teflon glass potter, the homogenate was filtered over two layers of cheesecloth. The filtrate was centrifuged at 20,000 × g for 15 min (GSA rotor, Sorvall centrifuge). The supernatant was filled into 70-ml polycarbonate centrifuge bottles (Beckman Instruments), and membrane fractions were collected by centrifugation at 80,000 × g for 1 h. The high speed supernatant was usually removed, the membrane pellet was resuspended in 25 mM Tris-MES, 3 mM MgCl2, pH 7.5, with a hair brush, and then was aliquoted and stored at −70°C until use. All steps were performed on ice. The protein content of microsomal preparations was determined according to Layne (1957) by measuring the optical density of the samples at 280 and 260 nm. The ratio of the values obtained (280/260) was used to determine the protein concentration. We usually obtained 10-15 mg of microsomal membranes from 100 g of cell fresh weight (22 × 106 cells); thus, 1 mg of microsomal membranes contained about 500 million (Cosio and Dixon, 1992).

**Purification of Elicitor-active Glycopeptides and Oligosaccharides**—Glycopeptide elicitors and invertase oligosaccharides were purified from α-chymotrypsin-cleaved yeast invertase (Boehringer, Mannheim, Germany) as described previously (Basse et al., 1992). gp 8 was further purified by HPLC (Beckman Gold, San Ramon, CA) on a C18 ultraprecoated column (5 μm, 4.6 × 250 mm, Beckman) eluting with a linear gradient from 0 to 22% (v/v) acetonitrile in 10 mM acetic acid adjusted to pH 6.0 with trimethylamine at a flow rate of 0.8 ml/min within 2 min. After an additional 8 min, a concave gradient from 22-20% (v/v) acetonitrile for 50 min was added, gp 8, a-d, eluted at 6.01, 6.35, 7.12, and 7.51% acetonitrile, respectively. Oligosaccharides isolated from yeast invertase were the same as described previously with structural confirmation by H NMR (Basse et al., 1992). Glycopeptides with large high mannose glycans were obtained from α-chymotrypsin-digested yeast invertase and were isolated in the void volume of a Sephadex G-75 (4 Bio-Rad, 3 × 600 mm) column (Fischer et al., 1992). The gp 8, α-chymotrypsin-digested yeast invertase, and were isolated in the void volume of a Sephadex G-75 (4 Bio-Rad, 3 × 600 mm) column (Fischer et al., 1992). The gp 8, α-chymotrypsin-digested yeast invertase, and were isolated in the void volume of a Sephadex G-75 (4 Bio-Rad, 3 × 600 mm) column (Fischer et al., 1992).

**Derivatization of gp 8—Derivatization of gp 8 with [35S]Boc-Met-OSu—Boc-Met-Osu (500 μCi), with a specific radioactivity of 930 Ci/mmol, supplied in 0.5 ml of benzene through Amersham, United Kingdom, was dried by evaporating the solvent under a gentle stream of N2. The dried product was then redissolved in NaB03, pH 8.5, and added to Boc-Met-Osu (80 μg). The reaction was allowed to proceed for 5 h on ice (A). gp 8 (20 μg) was dissolved in Me2SO (20 μl) and added to Boc-Met-Osu (2 μg). The reaction was allowed to proceed for 24 h at 37°C. Me2SO was subsequently removed by drying in a vacuum concentrator (speed vac) (B).

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**Derivatization of the Peptide Sequence—Amino acid sequencing was performed by automated Edman degradation using the method of Edman and Begg (1967).**

**Determination of the Carbohydrate Concentration—Carbohydrates were quantitatively measured by the phenol/sulfuric acid assay (Dubois et al., 1956).**

**Binding Assay—35S-Labeled gp 8c (~900 Ci/mmol, usually 3 × 108 cpm/0.1 ml corresponding to 1.5 nM or at the concentrations indicated) was incubated with membrane fractions containing 200 μg of protein either alone (total binding) or with appropriate concentrations of competitors in 100 μl of binding buffer (25 mM Tris-MES, pH 7.0, 3 mM MgCl2, 0.1 M NaCl, 0.1 mM methionine) on ice for 1.5 h. Assays were stopped by filtering the reaction mixture through a Whatman GF/B filter (2.5-cm diameter) previously soaked for 2 h in ice-cold binding buffer following by a wash with 10 ml of ice-cold binding buffer. The washed filters were transferred into plastic vials, scintillation fluid (3.5 ml each), and radioactivity was determined on a Beckman LS-9000 liquid scintillation analyzer. Specific binding was determined as the amount of radioactivity bound under the same conditions but in the presence of 0.5 μM unlabeled gp 8c (500-fold molar excess) and subtracted from the total binding to yield specific binding. The Kd and the maximal content of binding sites (Bmax) were calculated from ligand saturation data (Cantor and Kinetic studies were performed on ice. The protein content of microsomal preparations was determined according to Layne (1957) by measuring the optical density of the samples at 280 and 260 nm. The ratio of the values obtained (280/260) was used to determine the protein concentration. We usually obtained 10-15 mg of microsomal membranes from 100 g of cell fresh weight (22 × 106 cells); thus, 1 mg of microsomal membranes contained about 500 million (Cosio and Dixon, 1992).
Schimmig, 1980). Binding to whole cells was performed by incubation on a shaker at 4 °C of 1-ml suspensions of cultured tomato cells (6.2 g of cell fresh weight) supplemented with 20 μl of a saturated methionine solution and [35S]Met 8c at the indicated concentrations in the range of 3 x 10^6 cpm/ml (0.15 nM) in 2.5-ml Eppendorf tubes for 90 min. Unspecific binding was determined as the amount of radioactivity bound under the same conditions but in the presence of 0.05 μM unlabelled gp 8c (800-fold molar excess). Assays were stopped as described above with the modification that ice-cold fresh culture medium (Felix et al., 1991a) supplied with 10 nM methionine was used for preincubation of the filters and for the washing step. Binding to whole cells at room temperature was performed under the same conditions but with the further addition of 1 mg/ml bovine serum albumin digested with proteinase K (Boehringer) and boiled upon incubation. Radioactivity was determined by scintillation counting as described above.

RESULTS

Preparation of gp 8 and Derivatization with Boc-Met-OSu—As described previously (Basse et al., 1992), gp 8 is homogeneous with respect to the peptide sequence (Asn-Gly-Thr-His-Phe) but heterogeneous with respect to oligosaccharide chains, and the activity of the various glycopeptides critically depends on the size of the attached oligosaccharide chains. We separated gp 8 into 4 glycopeptides (gp 8, a–d, with 8–11 mannoses) by HPLC on a C18 reversed phase column eluting with a very shallow gradient. The elicitivities of gp 8, a–d, were compared based on their capacity to stimulate ethylene biosynthesis in tomato cells (see Table I, left column). Elicitor gp 8c (Mr 2620) induced ethylene biosynthesis half-maximally (EC50 value) at a concentration of 10 ng/ml based on carbohydrate equivalents, i.e., at 6 nM. Gp 8a (M, 2296) was a very inefficient elicitor with an EC50 value of >1000 ng/ml based on carbohydrate equivalents, i.e., >760

![Fig. 1. Chromatography of the reaction products of gp 8c incubated with nonradioactive Boc-Met-OSUs and [35S]Boc-Met-OSu. Reaction mixtures of gp 8c and Boc-Met-OSUs were separated by HPLC on a C18 ultraphase column. Thin line, absorption peaks (A214 nm) of a mixture of products generated by incubation of gp 8c (20 μg) with 2 μg of nonradioactive Boc-Met-OSu. Thick line, radioactivity of aliquots (2 μl) of the fractions (0.5 ml) obtained by chromatography of the reaction mixture of gp 8c (20 μg) and [35S]Boc-Met-OSu (500 μCi). The identity of peaks (A-E') is explained in the text.](https://example.com/fig1.png)
elicitor activity (half-maximal activity at 8 nM instead of 4 nM) (see Table I). In addition, gp 8c with the sequence Arg-Asn-Asp-Ser-Gly-Ala-Phe and gp 8b cleaved with endoproteinase Asp-N (Basse et al., 1992) were derivatized with Boc-Met sulfoxide. We generally used peak C (the methionine sulfoxide form of gp 8c) as compared with peak E (the methionine form of gp 8c) as reported in Basse and Boller (1992; Basse et al., 1992). The capacity of different microsomal membrane preparations to bind radioactively labeled ligand differed by a factor of 2. Unspecific binding was reduced by the addition of 10 mM methionine and 0.1 M NaCl to the assay mixture. Higher salt concentrations reduced binding, and binding was completely abolished at 1 M NaCl (not shown). In addition, specific binding was also abolished by heating microsomal membranes at 56°C for 10 min. Specific binding was highest at pH values in the range of 6-8 but was 50% reduced at pH 4, 5, and 9 and nearly prevented at pH 3 (not shown). A kinetic analysis demonstrated that association of 1.4 nM [35S]gp 8c to 200 µg of microsomal membranes was half-maximal at 40 min (Fig. 2). Further association was stopped by the addition of 0.5 µM gp 8c at 60 min and resulted in a rapid dissociation of bound [35S]gp 8c indicating reversible elici
tor activity in some but not all cases.

### Derivatization of gp 8c with [35S]Boc-Met-OSu—We have chosen the highly potent elicitor gp 8c to attach an N-terminal Boc-[35S]methionine group. Derivatization of gp 8c was performed with 500 µCi (540 pmol) [35S]Boc-Met-OSu (930 Ci/mmol). After incubation, the reaction mixture of gp 8c (20 µg) and [35S]Boc-Met-OSu (500 µCi) was separated by HPLC (see “Experimental Procedures”). Aliquots of the fractions were collected, and their radioactivity was determined (Fig. 1). Radioactivity in peaks A–E represented 26–32, 16–25, 15–22, 2–14, and 0.7–4.4%, respectively, of the initial [35S]Boc-Met-OSu, as determined from three separate derivatizations using different [35S]Boc-Met-OSu preparations. All radioactive peaks were tested for their ability to bind to concanavalin A. Peaks C and E were retained to about 90% by concanavalin A coupled to agarose, indicating that they both represented derivatized gp 8c (not shown). None of the other peaks bound to concanavalin A. Peak E (a minor peak in most preparations) could be identified as Boc-[35S]Met-8c by comparison with the absorption peaks (A214) of a mixture of products generated by incubation of gp 8c with a larger amount of nonradioactive Boc-Met-OSu (thin line in Fig. 1). As stated above, peak E' was shown to be the N-methionyl derivatized gp 8c by automated sequence analysis. We suspected peak C to be Boc-[35S]Met-sulfoxide-gp 8c. To test this, the labeled material eluting at 34 min (peak C) was reduced in 0.72 M β-mercaptoethanol. When this mixture was subjected to HPLC after increasing incubation times, more and more radioactivity was shifted to the position of peak E, reaching 25% of the radioactivity after 7 h and 54% after 24 h. Thus, the authentic Boc-methionyl-gp 8c could be generated from peak C, strongly suggesting that peak C contains an oxidized form of methionine, probably methionine sulfoxide. If it is taken into account that both peaks C and E represent similarly derivatized forms of gp 8c, the total yield of derivatized molecules was about 20%. By comparison with nonradioactive standards, peak D was identified as unreacted Boc-Met-OSu. Peak B probably represented the methionine sulfoxide form of Boc-Met-OSu. Peak A (retention time of 20 min) was identified as its hydrolysis product, Boc-methionyl sulfoxide by co-chromatography with 200 µg of nonradioactive Boc-Met-sulfoxide, monitoring radioactivity and absorption (A214). The retention time of nonradioactive Boc-Met standard was 31 min under the same conditions. This indicates that our starting material, when hydrolyzed, predominantly coeluted with Boc-Met-sulfoxide. We generally used peak C (the methionine sulfoxide form of gp 8c) for binding assays but obtained similar results with peak E (the methionine form of gp 8c) (not shown).

### Binding of [35S]gp 8c to Tomato Microsomal Membranes—Labeled elicitor [35S]gp 8c (peak C) was tested as ligand using a filtration assay with a membrane fraction of tomato cells and a technique similar to the one described by Cosio et al. (1988). The capacity of different microsomal membrane preparations to bind radioactively labeled ligand differed by a factor of 1–2. Unspecific binding was reduced by the addition of 0.5 µCi unlabeled gp 8c added at zero time (0). Each data point represents the average of two replicates.

**Fig. 2.** Time course and reversibility of binding of [35S]gp 8c to tomato microsomal membranes. Radioactivity bound at various times after addition of 0.14 nM [35S]gp 8c to 200 µg of microsomal membranes (○), after addition of 0.5 µCi unlabeled gp 8c at 60 min, dashed line (V), and in the presence of 0.5 µCi unlabeled gp 8c added at zero time (O). Each data point represents the average of two replicates.
efficient suppressors were the same as for the oligosaccharide side chains of the glycopeptides for high elicitor activity. This led us to assume that the glycan suppressors and the glycopeptide elicitors competed for the same binding site. To study this, we compared a number of glycans, glycopeptides, and purified yeast invertase with respect to their abilities to compete with [35S]gp 8c for specific binding (Fig. 5). We used the amount of carbohydrates in the preparation as a basis for comparison, since some of the preparations contained mixtures of molecules. A comparison of the elicitor-active gp 8c and the suppressor-active glycan Man12GlcNAc and of the elicitor-inactive gp 8a and the corresponding suppressor-inactive glycan Man8GlcNAc is shown in Fig. 5A. This revealed that the glycan Man12GlcNAc and gp 8c exhibited IC50 values (the value for half-maximal displacement) of 26 and 18 ng/ml, respectively (Table I). The IC50 value of Boc-Met-gp 8c was 1.5-fold higher compared with gp 8c (Fig. 5B). The IC50 values of the glycan Man8GlcNAc and gp 8a were 6008 and 1565 ng/ml, respectively (Table I). (The lower IC50 value of gp 8a as compared with Man8GlcNAc might possibly be due to a small contamination of gp 8a with gp 8b.) This shows that low elicitors, respectively suppressor activities, match with low affinities in the displacement assays, as seen from the high IC50 values. The results also demonstrate that glycopeptide elicitors and glycan suppressors compete with similar IC50 values for the same binding site in tomato microsomal fractions, indicating that the oligosaccharide isolated from an elicitor-active glycopeptide has the same binding affinity as the glycopeptide itself. The high concentrations of gp 8a required to displace binding of [35S]gp 8c match with the very low binding activity of [35S]gp 8a in the concentration range tested (Fig. 4B). The displacement studies were further extended to various preparations obtained from yeast invertase (Fig. 5, A and B). Purified yeast invertase both exhibited a more than 1000-fold lower elicitor activity and IC50 value when compared with gp 8c, demonstrating the inaccessibility of elicitor-active moieties on yeast invertase to the putative receptor. Cleavage of purified invertase by α-chymotrypsin resulted in 50–100-fold increased elicitor and binding activities. The IC50 values of gp 10 (Basse et al., 1992) with the sequence Lys-Ala-Glu-Pro-Ile-Leu-Asn-Ile-Ser-Asn-Ala-Gly-Pro-Trp and Boc-Met-gp59e were lower, i.e. their affinities higher than expected from their elicitor activities (Table I). Whereas the elicitor activity of gp 10 was more than 100-fold reduced in comparison with gp 8c, the IC50 value was only 18-fold reduced. A possible explanation for this discrepancy is a better accessibility of the ligand for its binding site on isolated membrane fractions compared with intact cells (Table I). Yeast cell wall glycoproteins are known to have two classes of N-linked oligosaccharides: a class of small high mannose glycans (Man6,12GlcNAc2) and a class of large polymannose
chains (reviewed by Tanner and Lehle, 1987). A preparation containing fragments with large polymannose chains obtained from α-chymotrypsin digested purified invertase exhibited a very low elicitor and displacement activity (Table I) emphasizing the preference of the elicitor binding site for small high mannose glycans. In order to determine the $K_i$ of the suppressor Man$_1$GlcNAc, a series of concentrations of this suppressor was assayed in combination with four different concentrations of [${}^{35}$S]gp 8c (Fig. 6A). In the same way, the $K_i$ value of suppressor Man$_1$GlcNAc was determined in vitro based on ethylene formation (Fig. 6B). Dixon plot analysis of the data yielded similar $K_i$ values for inhibition of elicitor binding and for suppression of elicitor activity (10 and 5 nM, respectively), suggesting that the suppressor exerts its activity in vivo by competition for the binding site revealed in vitro.

**Binding to Whole Cells**—We obtained specific binding of [${}^{35}$S]gp 8c incubated with whole suspension-cultured tomato cells at 4 °C and at room temperature, although unspecific binding was higher than with membrane fractions. Association of 0.11 nM [${}^{35}$S]gp 8c to cells was half-maximal after 60 min at 4 °C (Fig. 7A). Unspecific binding was determined in the presence of 50 nM gp 8c. This concentration was sufficient to abolish specific binding when added concomitantly with [${}^{35}$S]gp 8c (not shown). Addition of 50 nM gp 8c at 60 min completely prevented further binding of [${}^{35}$S]gp 8c and resulted in a slight dissociation of bound labeled ligand (Fig. 7A). In order to demonstrate binding under conditions in which cells are still responsive to elicitor, binding was performed at room temperature. Whereas ethylene formation could not be induced by elicitors at 4 °C, cells were responsive to elicitors at the exact conditions used for in vivo binding assays at room temperature (not shown). However, the ratio of total binding to the apparent nonspecific binding was ~2 at room temperature. The reason for this might be the presence of extracellular or cell wall-localized proteases resulting in release of [${}^{35}$S]methionine or derivatives from [${}^{35}$S]gp 8c, which might be taken up by the plant. A number of proteases have been described in plants (Boller, 1986) including tomato (Vera and Conejero, 1989; Rodrigo et al., 1991). To reduce nonspecific binding, a mixture of peptides obtained by partial hydrolysis of bovine serum albumin with proteinase K was added in the binding assay. This resulted in total binding, which was approximately 3-fold higher than nonspecific binding. A kinetic analysis of binding at room temperature showed that association of 0.2 nM [${}^{35}$S]gp 8c to whole cells was half-maximal after 60 min, and hence followed a similar kinetics
Specific Elicitor Binding Sites in Tomato

![Graph A](image1)

**FIG. 7.** Time course and reversibility of binding of [35S]gp 8c to whole cells at 4 °C (A) and 25 °C (B). Radioactivity bound various times after addition of 0.11 nM [35S]gp 8c (A) or 0.2 nM [35S] gp 8c (B) to whole cells (●), after addition of 0.05 μM unlabeled gp 8c at 60 min, (dashed line, ▲), and in the presence of 0.05 μM unlabeled gp 8c added at zero time (●). Each data point represents the average of two replicates.

![Graph B](image2)

**FIG. 8.** Kinetics of binding of [35S]gp 8c to whole cells. Increasing amounts of [35S]gp 8c were incubated with tomato cells at 4 °C as described under "Experimental Procedures" to determine total binding (●). Nonspecific binding was determined in the presence of 0.05 μM gp 8c (○). Each data point represents the average of two replicates.

As at 4 °C (Fig. 7B), Addition of 50 nM gp 8c to cells pretreated with [35S]gp 8c for 60 min reduced further binding and resulted in dissociation of bound labeled ligand within 3 h after the addition of competitor (Fig. 7B). Saturation of binding to whole cells was reached at about 1 nM [35S]gp 8c (Fig. 8) at 4 °C. An estimation of the binding data obtained by Scatchard analysis indicated a Kd value of 0.7 nM and a Bmax value of 60 fmol/g cell fresh weight (not shown). This value corresponds to approximately 1.5 × 106 binding sites/cell. The dissociation kinetics of [35S]gp 8c (see Fig. 7) was compared with decay of ACC synthase activity (Fig. 9). ACC synthase is the key enzyme in ethylene biosynthesis (Yang and Hoffman, 1984). When a 40-fold excess of suppressor ManαGlcNAc was added to cells pretreated with elicitor gp 8c for 150 min, ACC synthase activity was slightly stimulated compared with the water control and further increased for 20 min but then decreased with a half-time of 20 min, indicating that ACC synthase was rapidly inactivated when the suppressor replaced the elicitor at the binding site. The suppressor-inactive glycan ManαGlcNAc also slightly stimulated ACC synthase but did not cause a premature inactivation of ACC synthase. This supports the notion that continuous presence of elicitor at the binding site is necessary for full induction of ethylene biosynthesis in suspension-cultured tomato cells (Felix et al., 1991), and it suggests that prevention of elicitor binding to tomato cells leads to rapid loss of the induced state.

**FIG. 9.** ACC synthase activity in tomato cells treated with elicitor and suppressor. Tomato cells (30 ml) were shaken in 100-ml flasks, and 1-ml samples were removed at the time points indicated for determination of ACC synthase activity. Cells were treated with elicitor gp 8d (150 ng/ml) at zero time, and the following substances were added at the time indicated by the arrow: ManαGlcNAc (6 μg/ml) (V), ManβGlcNAc (6 μg/ml) (Δ), or water (●). As a control treatment, ManαGlcNAc (6 μg/ml) was added concomitantly with the elicitor (○). Dotted line, water control (□).

**Discussion**

Our report clearly demonstrates the existence of specific elicitor binding sites in tomato cells. We have shown specific, saturable, and reversible binding of [35S]-labeled glycopeptide elicitors to whole tomato cells and microsomal membranes. Elicitor binding sites are known from soybean where their occurrence was demonstrated in membranes and protoplasts (Schmidt and Ebel, 1987; Cosio et al., 1988, 1990, 1992; Cheong and Hahn, 1991). Here, we approach in vivo conditions even more, demonstrating specific binding of radiolabeled elicitors to whole cells, both at 4 °C and under conditions in which cells are still responsive to elicitors. We observed specific binding of [35S]gp 8c to whole cells at 4 and 25 °C indicating the presence of a surface-localized binding site. Specific binding to whole cells was suppressed by the addition of gp 8c, and dissociation of bound labeled ligand was observed both at 4 and 25 °C indicating reversible binding and an absence of internalization of labeled elicitor by the cells. This argues against the hypothesis of Horn et al. (1989) who proposed receptor-mediated uptake of elicitors based on accumulation of fluorescence in the vacuoles upon incubation of suspension-cultured soybean cells with fluorescein-labeled elicitor at 23 but not 4 °C.

Ligand saturation experiments performed with microsomal membranes gave a Kd value of 3.3 nM for [35S]gp 8c as determined by Scatchard analysis indicating a high affinity.
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binding site on microsomal membranes. Binding of \[^{35}S\]gp 8c to microsomal membranes was reversible, and dissociation of bound labeled ligand was more rapid than with whole cells, possibly because of (i) the higher concentration of competitor used for displacement of radiolabeled elictor from microsomal membranes and (ii) the better accessibility of competitor to its binding site on microsomal membranes compared with whole cells. Scatchard analysis of the binding data obtained with microsomal membranes also revealed a \(B_{max}\) value of 19 fmol/mg protein corresponding to \(2.5 \times 10^3\) binding sites on a single cell. This is probably an underestimation due to the yield of microsomal membranes obtained from suspension-cultured tomato cells and the inaccessibility of binding sites trapped within membrane vesicles. Indeed, the binding study with whole cells indicated a \(B_{max}\) value of 60 fmol/g cell fresh weight, corresponding to \(1.6 \times 10^3\) binding sites/cell. The low \(K_d\) value of 3.3 nM indicates a similar high affinity binding site for the yeast-derived glycopeptides in tomato cells as found with the \(^{125}\)I-labeled hepta-\(\beta\)-glucoside in soybean (Cosio et al., 1990; Cheong and Hahn, 1991) and clearly distinguishes the putative elictor receptor from lectins, proteins of nonimmune origin that bind oligosaccharides with binding constants in the range of \(10^6\) to \(10^7\) M\(^{-1}\) (reviewed by Sharon and Lis, 1989). The Hill coefficient of approximately 1 (see Fig. 3C) excludes cooperative binding, and the linearity of the Hill plot suggests a single class of elictor binding sites. Identical characteristics were attributed to the hepta-\(\beta\)-glucoside binding site (Cheong and Hahn, 1991).

There is a high degree of correlation between the activity of the yeast invertase-derived elictors and suppressors, respectively, and their displacement activities (IC\(_{50}\) values). This was demonstrated with various elictor-active components and the glycans Man\(_{10}\)GlcNAc and Man\(_{9}\)GlcNAc (see Table I). We infer from the results that the elictor recognition site has a high affinity to small high mannose oligosaccharides consisting of more than 8 mannosyl residues. Elicitor gp 8c and the glycan Man\(_{11}\)GlcNAc exhibited very similar IC\(_{50}\) values, and the \(K_d\) value calculated for \[^{35}S\]gp 8c was very similar to the \(K_d\) value calculated for the suppressor Man\(_{11}\)GlcNAc. This provides evidence that the elictor-active glycopeptides and suppressor-active glycans derived from these elictors compete with similarly high affinities for the same binding site and makes a cooperative effect of the peptide moiety on glycans binding unlikely. No detectable specific binding was obtained with the \(^{35}S\)-labeled peptide released from gp 8c by Endo H, and binding of \[^{35}S\]gp 8a was strongly reduced compared with \[^{35}S\]gp 8c emphasizing the decisive role of the glycan moiety for binding activity. However, the peptide moiety is absolutely required for elictor activity of the yeast-derived glycopeptides (Basse and Boller, 1992; Basse et al., 1992). It is conceivable, therefore, that the presence of a short peptide attached to the chitinobiose at the reducing end of the glycan might contribute to a conformational change of the receptor required for turning on signal transduction (Grosskopf et al., 1990; Felix et al., 1991b). The requirements of the peptide moiety for elictor activity of the glycopeptides have previously been analyzed (Basse et al., 1992). This had indicated that the peptide moiety of gp 38 (see above) could be reduced to Arg-\(\alpha\)-Asn without affecting the elictor activity. The exact molecular structures of the peptide moiety required for elictor activity remain to be elucidated.

Our results provide a first example of a class of glycopeptides that act as agonists but can be converted into antagonistic glycans, and it will be exciting to explore the biochemical and genetic bases for this intriguing mechanism.

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