The Plant Wound Hormone Systemin Binds with the N-Terminal Part to Its Receptor but Needs the C-Terminal Part to Activate It

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Suspension-cultured cells of Lycopersicon peruvianum respond with rapid medium alkalinization and a strong increase of a MAP kinase-like activity when treated with subnanomolar concentrations of the plant wound hormone systemin. Systemin fragments comprising the N-terminal 14 amino acids (syst<sup>1-14</sup>) or the C-terminal four amino acids (syst<sup>15-18</sup>), added singly or in combination, were inactive as inducers of these responses. Syst<sup>1-14</sup> but not syst<sup>15-18</sup> antagonized activity of intact systemin in a competitive manner. Likewise, intact systemin showed stimulatory, syst<sup>1-14</sup> antagonistic activity, and syst<sup>15-18</sup> showed no activity in leaf pieces of tomato (L. esculentum) plants assayed for the induction of ethylene biosynthesis. To study the molecular basis of perception, we extended the C-terminal end of systemin by a tyrosine residue and radioiodinated it to yield systemin-<sup>125</sup>I-iodotyrosine. In membrane preparations of L. peruvianum, this radioligand exhibited rapid, saturable, and reversible binding to a single class of binding sites. Binding showed a dissociation constant of ~1 nM, and binding of radioligand was efficiently competed by unlabeled systemin but not by syst<sup>1-14</sup> or structurally unrelated peptides. Binding was also competed by the systemin antagonists syst<sup>1-14</sup> and syst-Ala-17 (IC<sub>50</sub> of 500 and 1000 nM, respectively). Thus, this binding site exhibits the characteristics expected for a functional systemin receptor. Based on these results, we propose a two-step mechanism for systemin action, with binding of the N-terminal part to the receptor as the first step and activation of responses with the C-terminal part as the second step.

INTRODUCTION

Plants react to localized, mechanical wounding with a variety of responses (Chessin and Zipf, 1990). Wounding of tomato leaves results in the synthesis of proteinase inhibitor proteins at the site of wounding but also in distant, unwounded leaves (Ryan, 1992), indicating that a wound signal spreads systemically throughout the plant. The search for chemical signals in wound exudates that have the capacity to induce proteinase inhibitors in unwounded tissue led to the discovery of the 18–amino acid peptide systemin (Pearce et al., 1991). This peptide, released from a 200–amino acid pro-systemin protein (McGurl et al., 1992), induces proteinase inhibitors when applied at femtomole doses per plant. A radioactively labeled peptide applied to wound sites is transported throughout the plant (Pearce et al., 1991). This peptide, released from a 200–amino acid pro-systemin protein (McGurl et al., 1992), induces proteinase inhibitors when applied at femtomole doses per plant. A radioactively labeled peptide applied to wound sites is transported throughout the plant (Pearce et al., 1991). Plants expressing antisense mRNA for prosystemin have an impaired systemic wound response, and plants overexpressing sense mRNA constitutively synthesize proteinase inhibitors (McGurl et al., 1992, 1994). Although these results provide strong evidence for the role of systemin as a wound hormone, they do not rule out electrical and hydraulic processes associated with the systemic spreading of the wound signal (Wildon et al., 1992; Malone et al., 1994).

Responses to systemin in the target cells appear to be mediated by induction of the octadecanoid pathway (Conconi et al., 1996) and the activation of a 48-kD myelin basic protein (MBP) kinase (Stratmann and Ryan, 1997). However, little is known about the initial steps of systemin perception by these target cells. Analysis of structure–activity relationships of deleted or substituted systemin molecules has indicated that the C-terminal part of the molecule is both necessary and sufficient for biological activity (Pearce et al., 1993). The high sensitivity and structural selectivity of tomato for these presumably membrane-impermeable oligopeptide signals have suggested perception by a specific plasma membrane receptor with an affinity for the C terminus of systemin. Subsequently, a binding site for systemin was indeed detected on plasma membranes of tomato cells (Schaller and Ryan, 1994). However, binding was associated with proteolytic cleavage of the peptide, and the binding site had affinity only for the N-terminal part of systemin. Therefore, this binding site was thought to be involved in degradation of systemin rather than being a receptor.

Previously, we described rapid responses of suspension-cultured Lycopersicon peruvianum cells to treatment with subnanomolar concentrations of systemin (Felix and Boller, 1998).
These responses resembled the responses described for many tissue-cultured cells treated with microbial-derived elicitors (Boller, 1995) and included medium alkalinization, a concomitant efflux of K⁺, increased biosynthesis of the stress hormone ethylene, and stimulation of phenylalanine ammonia-lyase.

Perception of systemin in these L. peruvianum cells resembles perception of this signal molecule in tomato plants with regard to specificity and sensitivity. In this study, we used these cells as a convenient experimental model to study the molecular mechanism of systemin perception. With a highly sensitive binding assay that uses a radiiodinated derivative of systemin, we discovered a novel, specific, high-affinity binding site in membrane preparations of L. peruvianum. We demonstrate that binding activity of different systemin derivatives is closely correlated with their biological activity, indicating that this binding site acts as the functional receptor of systemin.

RESULTS

Alkalinization of Culture Medium in Response to Systemin Fragments and Derivatives

Induction of proteinase inhibitors in tomato leaves has been examined extensively with regard to the structure-activity relationship of systemin-related peptides as inducers (Pearce et al., 1993). The smallest peptide that still retained at least weak systemin activity was identified as the tetrapeptide MQTD (named syst15–18 here), comprising the four C-terminal amino acid residues of systemin. Suspension-cultured cells of the wild tomato species L. peruvianum react to subnanomolar concentrations of intact systemin with rapid alkalinization of the extracellular medium (Felix and Boller, 1995; Figure 1). When treated with saturating doses of systemin (>0.3 nM), the extracellular pH remained constant for 1 min and then rapidly increased by more than one pH unit. In contrast, the C-terminal tetrapeptide syst15–18 did not induce significant alkalinization at concentrations tested up to 30 μM (example for 10 μM shown in Figure 1). Furthermore, no alkalinization response was observed with the peptide comprising the N-terminal 14 amino acid residues (syst1–14) or with syst1–14 and syst15–18 added in combination (Figure 1). Apparently, the systemin peptide must be intact to induce L. peruvianum cells. We also tested the two systemin fragments in combination with systemin and observed that the alkalinization induced by intact systemin was completely blocked by the N-terminal fragment, syst1–14. The C-terminal fragment, syst15–18, had no inhibitory activity (Figure 1).

The suppressive effect of syst1–14 was competitive and could be overcome by increasing concentrations of systemin. When we used the extracellular pH after 15 min of treatment as a bioassay (Felix and Boller, 1995), 10 μM syst1–14 shifted the concentration of systemin that caused a half-maximal induction (EC50) from 0.1 to ~10 nM (Figure 2). Syst1–14 acted as a specific antagonist of systemin and did not inhibit the alkalinization response induced by unrelated elicitors, such as chitin fragments (data not shown). The addition of 10 μM syst15–18, in contrast, had no inhibitory effect. The slight synergistic effect apparent in the experiment shown in Figure 2 could not be observed in repetitions of this experiment (data not shown).

Induction of Responses in Tomato Leaf Tissues

We also tested systemin and the systemin fragments for the induction of rapid responses in tissues of L. esculentum.
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Plants. Leaf slices from *L. esculentum* plants did exhibit alkalinization in response to systemin treatment. Due possibly to the small number of accessible cells at the surface of the tissue and/or to the relatively high extracellular pH of 6.5 in untreated controls, this pH increase was very small (<0.2 pH units) and could not be observed in every experiment (data not shown). In contrast, the leaf slices consistently responded with rapid, easily measurable induction of ethylene biosynthesis when treated with systemin. In the experiment shown in Figure 3, ethylene produced during the first 2 hr of treatment with 10 nM systemin was 10 times higher than in mock-treated controls. Treatment with 10 µM syst1-14, syst15-18, or syst1-14 and syst15-18 did not induce ethylene production. Syst1-14, but not syst15-18, acted as an inhibitor of systemin-induced ethylene biosynthesis (Figure 3). A similar suppression of ethylene production also was observed with the systemin antagonist syst-Ala-17, a systemin analog with Thr-17 replaced by Ala (Pearce et al., 1993). Suppression by syst1-14 and syst-Ala-17 was specific for systemin, and induction by unrelated stimuli, such as xylanase from *Trichoderma viride*, was not affected (data not shown).

**Induction of MBP Kinase Activity**

Recently, rapid activation of a 48-kD MBP kinase has been reported in leaves of young tomato plants after wounding or treatment with systemin (Stratmann and Ryan, 1997). Therefore, we tested whether systemin would stimulate activity of an MBP kinase in *L. peruvianum* cells in a similar manner. An MBP kinase migrating with an apparent molecular mass of ~48 kD after SDS-PAGE was rapidly and strongly induced after treatment with 0.3 nM systemin (Figure 4A). Activation was visible after 2 min of treatment and reached a plateau within 4 min (Figure 4B). Neither syst1-14 nor syst15-18, added at concentrations of 30 µM, activated this MBP kinase. Syst1-14, but not syst15-18, had a suppressive effect and completely inhibited induction of the MBP kinase by 0.3 nM intact systemin (Figures 4A and 4B).

**Tyrosine Derivatives of Systemin**

To study binding sites for systemin, a radiolabeled systemin derivative with a high specific radioactivity was required. Systemin contains no Tyr residue that would allow direct radioiodination. Adding a Tyr residue at the N terminus led to a peptide, Tyr-syst, that had >1000-fold lower capacity to induce alkalinization in *L. peruvianum* cells (Figure 5). Coupling of a methionine group, a biotin group, or a fluorescent dye to the N terminus led to similar decreases in biological activity (data not shown).

In contrast, the C-terminal extension of the peptide with tyrosine (syst-Tyr) affected biological activity much less. The EC50 for the induction of alkalinization was only approximately sevenfold higher than that for unmodified systemin (Figure 5). Iodination of syst-Tyr by chloramine-T and KI (non-radioactive) did not change activity significantly (Figure 5).

![Figure 2](image2.png)

**Figure 2.** Competitive Antagonism of Syst1-14.

Shown is the alkalinization response of cells treated with different concentrations of systemin in the presence of 10 µM syst1-14 (closed circles) or 10 µM syst15-18 (open triangles). Controls were treated with systemin alone (closed diamonds). Extracellular pH was measured after 15 min of treatment.

![Figure 3](image3.png)

**Figure 3.** Induction of Ethylene Biosynthesis in Tomato Leaves.

Ethylene production was measured after 2 hr of treatment with (+) and without (−) systemin, syst1-14, syst15-18, and syst-Ala-17, as indicated at the bottom. Bars and error bars indicate mean and standard deviation of four replicates per treatment.
Binding of \(^{125}\)I-Labeled Syst-Tyr to Microsomal Membranes

A radiiodinated derivative of syst-Tyr, syst-Tyr–\(^{125}\)I, with a specific radioactivity of \(>2000\) C/mmol was used in binding studies with microsomal membrane preparations of \(L.\) peruvianum. Incubation of microsomal protein (100 \(\mu\)g) at 4°C with 76 fmol of radioligand resulted in rapid binding (Figure 6A). Association occurred with pseudo-first order kinetics; by fitting data to a monoeponential equation or by linearization, as shown in Figure 6B, a rate constant of \(k_{\text{obs}} = 0.15\) min\(^{-1}\) was estimated (half-maximal binding at 4.7 min; \(k_{12} = 1.15 \times 10^8\) [M\(^{-1}\) min\(^{-1}\)]). Maximal binding was reached within 20 min and remained stable for up to 3 hr. Nonspecific binding, assayed by adding 1 \(\mu\)M unlabeled syst-Tyr, remained low throughout the experiment (Figure 6A). Binding was fully reversible, and dissociation of syst-Tyr–\(^{125}\)I, induced by the addition of 1 \(\mu\)M unlabeled syst-Tyr after 20 min of incubation, occurred rapidly with first order kinetics and a half-life of 21 min (Figure 6B, \(k_{21} = 0.033\) min\(^{-1}\)). From this analysis of binding kinetics, a dissociation constant \(K_d = k_{21}/k_{12} = 0.29\) nM was estimated.

Kinetics of association and dissociation at 20°C were much faster. However, binding of the radioligand reached a maximum after 7 to 12 min and then decreased slowly (data not shown). No stable binding equilibrium was reached, probably because of inactivation of the radioligand, the binding site, or both.

At 4°C, binding of syst-Tyr–\(^{125}\)I to microsomal membrane preparations of \(L.\) peruvianum cells was saturable (Figure 7A). Fitting data of specific binding to a hyperbolic curve (Figure 7A, solid line) resulted in a \(K_d\) of 0.8 nM and a \(B_{\text{max}}\) of 59 fmol mg\(^{-1}\) microsomal protein. Scatchard analysis of the data showed similar values (Figure 7B; \(K_d\) of \(~1.1\) nM; \(B_{\text{max}}\) of \(~67\) fmol mg\(^{-1}\) protein) and indicated a single class of binding site. The Hill coefficient derived from these data was \(~0.95\) (Figure 7C). In summary, these saturation data show that systemin binds in a noncooperative manner to a single,
high-affinity binding site present at low abundance in the membranes of *L. peruvianum*.

**Specificity of Binding**

Specificity of binding was tested in competitive binding assays with increasing concentrations of unlabeled systemin and systemin derivatives. Examples are shown in Figure 8 for systemin, syst-Tyr, syst1–14, syst15–18, syst–Ala-17, and an unrelated peptide representing a domain of bacterial flagellin. The concentrations required to reduce binding of the radioligand by 50% (IC₅₀) were approximated from these curves and from similar competition experiments with several other peptides. In Table 1, these IC₅₀ values are compared with the relative capacities of these peptides to induce a half-maximal alkalinization response (EC₅₀). Unmodified systemin and syst-Tyr, which are the peptides with the highest capacities to induce alkalinization, were also the most efficient in competition of syst-Tyr–¹²⁵I in binding assays (IC₅₀ of 2 and 5 nM, respectively). Met-syst and Tyr-syst have a reduced capacity to induce alkalinization, and these peptides were also less efficient in competitive binding (IC₅₀ of 300 nM for both). Syst¹⁵–¹⁸ had no biological activity and did not significantly compete binding in concentrations up to 100 µM (IC₅₀ >> 100 µM). Syst¹⁴ and syst–Ala-17, which both behave as
antagonists of systemin, were found to compete for binding
with IC\textsubscript{50} values of 500 and 1000 nM, respectively. The unre-
related flagellin peptide, which is itself a potent inducer of the alkalini-
zation response in L. peruvianum cells (G. Felix, J. D. Duran, and T. Boller,
unpublished data), did not compete with binding of syst-Tyr\textsuperscript{125I} (IC\textsubscript{50} >> 100 \mu M).

DISCUSSION

Induction of proteinase inhibitors in leaves of young tomato
plants served as a bioassay for the original identification of
systemin (Pearce et al., 1991). More recently, induction of a MAP
kinase–like activity has been described that clearly
precedes induction of proteinase inhibitor genes after
wounding or treatment with systemin (Stratmann and Ryan,
1997). A similar kinase activity, detected with MBP as a sub-
strate by in-gel kinase assays, has been described after wounding or cutting in a variety of monocotyledonous and
dicotyledonous plant species (Seo et al., 1995; Usami et al.,
1995; Bögre et al., 1997). In all of these cases, activation
was detectable within minutes. Cytoplasmic phospholipase
A\textsubscript{2}, an enzyme that could release intermediates of the octa-
decanoic pathway from membranes, was postulated as a bi-
ological substrate for this kinase activity (Seo et al., 1995). In
this study, we observed that systemin induced a protein ki-
nase activity in L. peruvianum cells in a similar manner (Fig-
ure 4). This kinase resembled the kinase described for
tomato plants with regard to its apparent molecular mass in

Figure 7. Saturation of Syst-Tyr\textsuperscript{125I} Binding to L. peruvianum Membranes.

(A) Various amounts of syst-Tyr\textsuperscript{125I} diluted with nonradioactive syst-Tyr to a specific radioactivity of 22 Ci/mmol were incubated with membrane
fractions (110 \mu g of microsomal protein per assay) at 4°C for 30 min in the absence (total binding) or presence of 10 \mu M nonradioactive syst-Tyr
(nonspecific binding). Specific binding, determined by subtracting nonspecific binding from total binding, was fitted to a hyperbolic curve (solid
line).

(B) Scatchard analysis of data obtained for specific binding in (A).

(C) Hill plot of data obtained for specific binding in (A). Hill coefficient \(n_H\) is derived from the slope of the curve.
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the in-gel assays (48 kD) and the kinetics of induction, indicating that transduction of the systemin signal in tomato leaves and in cell cultures of *L. peruvianum* involves common elements.

Perception of systemin by cells of *L. peruvianum* also resembled perception in tomato with regard to the structure–activity relationship of the systemin signal. In both systems, the C-terminal part of the peptide is crucial for biological activity. Shortening the peptide at its C-terminal end (syst1–17 in Pearce et al. [1993] or syst 1–14 in this study) or replacing Thr-17 by an Ala residue (syst–Ala-17) led to loss of inducing activity. Although agonistic activity is lost in these systemin derivatives, they act as antagonists in both *L. peruvianum* and tomato, and they can suppress activity of authentic systemin in a competitive manner. Shortening or modifying the systemin peptide at its N-terminal end has been described as having more gradual effects on its activity in tomato plants (Pearce et al., 1993). In our bioassays, coupling an additional amino acid (Figure 5 and Table 1) or a fluorochrome (data not shown) to the N-terminal amino group did not completely abolish activity of systemin but led to a 500- to 1000-fold reduction in activity.

Syst15–18 did not induce medium alkalinization or MBP kinase activation in *L. peruvianum* cells, and it did not induce ethylene biosynthesis in tomato leaf tissue (Figures 1, 3, and 4). Thus, the four C-terminal residues of systemin appear to be essential but not sufficient for biological activity in our assays. This is in contrast to the earlier work of Pearce et al. (1993), who found a small inducing effect of syst15–18, albeit with an EC₅₀ 333,000-fold higher than that of intact systemin, leading them to conclude that the C terminus is both necessary and sufficient for activity. We cannot explain this apparent discrepancy, but responses studied in this work were measured after short treatments (up to 20 min for medium alkalinization and MBP kinase activation in *L. peruvianum* cells and 2 hr for the induction of ethylene biosynthesis in tomato leaf tissue) compared with the induction of proteinase inhibitors after 24 hr (Pearce et al., 1993).

Syst–Ala-17 has been described as an antagonist for systemin in tomato plants (Pearce et al., 1993), and it acts also as an antagonist in all of the bioassays used in our studies (Felix and Boller, 1995; this report). However, in addition, syst–Ala-17 exhibits partial agonist activity with regard to induction of alkalization (Felix and Boller, 1995). Partial agonistic activity is not uncommon for structural analogs acting as antagonists (Fletcher et al., 1993; Hibert et al., 1993), but syst–Ala-17 is somewhat peculiar because it shows weak agonistic activity only for a subset of responses. In preliminary experiments, we observed that 30 μM syst–Ala-17 also acted as a partial agonist for the induction of MBP kinase and induced <20% of the activity induced by 0.3 nM systemin (T. Meindl, data not shown). When added in combination with systemin, syst–Ala-17 had a suppressive effect and reduced the activity to the values observed after treatment with syst–Ala-17 alone. Although further experiments are needed to elucidate the mode of action of syst–Ala-17, this

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**Figure 8.** Competition of Syst-Tyr-125I Binding by Different Peptides.

Syst-Tyr-125I (0.4 nM) was mixed with various concentrations of unlabeled systemin, syst-Tyr, syst1–14, syst15–18, or syst–Ala-17 and incubated with membrane fractions of *L. peruvianum* (200 μg of microsomal protein per assay). Results were obtained with different batches of membrane preparations and are presented as the percentage of specific binding. Total binding for these different batches was between 4000 and 6000 cpm, and nonspecific binding was between 400 and 800 cpm, respectively.
opens the possibility that thresholds of intermediary signals, such as a MAP kinase activity or an intracellular change caused by the K⁺/H⁺ exchange across the plasma membrane, are required for the induction of the later responses.

Considering the importance of the C-terminal part of systemin for its biological activity (Pearce et al., 1993; Figures 1, 3, and 4), it was somewhat surprising that C-terminal extension of the peptide by an additional Tyr residue affected activity only marginally (<10-fold; Figure 5). Exact trimming of prosystemin at the C terminus (McGurl et al., 1992) does not appear to be essential for its activity. We also observed nearly full activity for a systemin peptide extended at its C terminus with the four amino acid residues present in the prosystemin polypeptide (data not shown). Because syst-Tyr also retained its activity after iodination of the Tyr residue (Figure 5), we used this derivative to produce a radiolabeled ligand. Binding studies with syst-Tyr–[125I] detected saturable, specific, and reversible binding to membrane preparations from L. peruvianum cells (Figures 6 to 8 and Table 1). This binding site had a high affinity for systemin. The $K_d$ derived from saturation data was ~1.1 nM (Figure 7), and a similar value of ~0.3 nM was estimated from kinetic analysis of association and dissociation of the radioligand (Figure 6).

### Table 1. Biological Activities and Binding Affinities of Systemin, Systemin Fragments, and Systemin Derivatives

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Biological Activity (EC₅₀) (nM)</th>
<th>Binding Affinity (IC₅₀) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systemin</td>
<td>0.06</td>
<td>2</td>
</tr>
<tr>
<td>Syst-Tyr</td>
<td>0.4</td>
<td>5</td>
</tr>
<tr>
<td>Tyr-syst</td>
<td>100</td>
<td>300</td>
</tr>
<tr>
<td>Met-syst</td>
<td>600</td>
<td>300</td>
</tr>
<tr>
<td>Syst-Ala-17</td>
<td>NM</td>
<td>1,000</td>
</tr>
<tr>
<td>Syst1–14</td>
<td>$&gt;$30,000</td>
<td>500</td>
</tr>
<tr>
<td>Syst15–18</td>
<td>$&gt;$30,000</td>
<td>$&gt;$100,000</td>
</tr>
<tr>
<td>Unrelated peptide from flagellin</td>
<td>(0.06)</td>
<td>$&gt;$100,000</td>
</tr>
</tbody>
</table>

a Concentration required for induction of half-maximal alkalization response in L. peruvianum cells. Results shown represent average values obtained in different experiments with different batches of cells.

b Concentration required to reduce binding of syst-Tyr–[125I] to microsomal membranes by 50%.

c NM, not measurable. Syst-Ala-17, besides acting as a competitive antagonist with an estimated $K_i$ of ~50 nM, shows characteristics of a partial agonist for induction of alkalization (Felix and Boller, 1995). Alkalization in response to syst-Ala-17 was weak, and maximal pH increase induced at saturating concentrations ($>$100 nM) was 0.45 pH units compared with 1.2 pH units observed with authentic systemin in the same batch of cells.

d Structurally unrelated peptide derived from bacterial flagellin that shows alkalization-inducing activity in a variety of cell cultures (G. Felix, J. D. Duran, and T. Boller, unpublished results).

A half-maximal alkalization response occurred at a concentration of ~0.4 nM syst-Tyr, a concentration close to the $K_d$ of the binding site. Thus, there seems to be a direct occupation-response relationship for induction of the alkalization response. Binding was specific and could be efficiently competed with systemin and syst-Tyr ($IC_{50} = 2$ and 5 nM, respectively) and, at higher concentrations, with the systemin antagonists syst-Ala-17 and syst15–18. No competition of binding was observed with syst15–18 or an unrelated peptide derived from flagellin protein. Tyr-syst and Met-syst, systemin derivatives with lower biological activities, were also weaker competitors for binding ($IC_{50}$ of 300 nM for both).

In a previous attempt by Schaller and Ryan (1994) to detect specific binding sites for systemin in membrane preparations of tomato leaves, systemin could be cross-linked specifically to a protein with an apparent molecular mass of 50 kD (SBP50) after SDS-PAGE. Interestingly, this binding had affinity for the N-terminal part of the peptide, and the systemin-derived peptide cross-linked to SBP50 lacked the C-terminal part. Cleavage of the peptide appeared to be necessary for binding to SBP50, and cleavage could be attributed to the presence of a furinlike protease activity present in these membranes (Schaller and Ryan, 1994). In our experiments, we used a systemin derivative labeled at its C-terminal end (syst-Tyr). This derivative would not allow the detection of binding of the N-terminal part after the removal of its C terminus. Thus, the binding site described in this work, although it also has affinity for the N-terminal part of systemin, does not depend on peptide cleavage. Association of systemin with SBP50 was slow and occurred only after >12 hr. In contrast, the binding described in this study was fast and occurred within minutes (Figure 6). This fast association is in good agreement with the kinetics of biological responses observed: after a lag phase of 1 min for medium alkalization (Figure 1) and after 2 to 5 min for induction of MBP kinase activity in L. peruvianum cells (Figure 4) and tomato plants (Stratmann and Ryan, 1997).

The binding site for systemin exhibits affinity for the N-terminal rather than the C-terminal part of the systemin molecule. Nevertheless, the C-terminal part is crucial for induction of biological responses. Perception of systemin is thus reminiscent of perception of fungal-derived glycopeptide elicitors in tomato cells (Basse et al., 1992). The intact glycopeptides act as potent elicitors of ethylene biosynthesis. Glycans released from the peptides are inactive as inducers but act as competitive suppressors and also efficiently compete with the intact glycopeptides for the single class of specific, high-affinity binding site present on tomato membranes. For systemin, we propose a similar mechanism for perception with binding of the N-terminal part, a conformational change or “induced fit” of the binding site, and activation of the receptor by the C-terminal part of systemin.

In summary, the binding site described in this work exhibits the characteristics expected for a functional systemin receptor. The L. peruvianum cells used in this study provide
an experimental system to study the systemin receptor and to dissect the molecular mechanism of signaling in the systemin response.

METHODS

Systemin Preparations and Radioiodination

The 18-amino acid peptide systemin (Pearce et al., 1991), the systemin fragments, and the systemin analogs were synthesized by F. Fischer (Friedrich Miescher-Institute). For (nonradioactive) iodination, 50 nmol of syst-Tyr in 20 μL of 10 mM Mops, pH 7.5, containing 25 mM KI was reacted with one chloramine-T bead (Fluka, Buchs, Switzerland) for 60 sec at 20°C. Systemin-3-I-iodotyrosine (syst-Tyr-I) was purified from the reaction mixture by HPLC on a C18 reversed-phase column. Syst-Tyr was radioiodinated with lactoperoxidase and 125I-iodine to yield systemin-3-125I-iodotyrosine (syst-Tyr-I25) of >2000 Ci/mmol by Anawa Trading SA (Wangen, Switzerland).

Cell Cultures

The cell suspension of Lycopersicon pereuvianum was kindly provided by L. Nover (University of Frankfurt, Germany). Cells were subcultured at 1-week intervals by diluting 3 mL of the suspension in 75 mL of fresh Murashige-Skoog-type medium supplemented with 5.0 mg L-1 1-naphthylacetic acid as described by Nover et al. (1982). Cells were grown on a rotary shaker at 26°C.

Measurement of Medium Alkalization

Aliquots of the cell suspension, 5 to 8 days after subculture, were incubated in open flasks on a rotatory shaker at 120 cycles per minute. Extracellular pH was measured with a small combined glass pH electrode (Metrohm, Herisau, Switzerland), and continuous measurements were registered using a pen recorder. Alternatively, extracellular pH was measured after 15 min of treatment.

In-Gel Protein Kinase Assay

Samples (200 μL) of L. pereuvianum cell cultures were collected at the time points indicated, mixed with an equal volume of 10% trichloroacetic acid, and frozen in liquid nitrogen. After thawing and ultrasonication, pellets were collected by centrifugation (10 min at 12,000 g) and washed twice with 80% acetone and 20% Tris-Mes buffer (20 mM; pH 8.0). Proteins were solubilized from pellets with SDS-sample buffer and separated by SDS-PAGE on gels containing 12% (w/v) acrylamide and 0.2% (w/v) MBP (Sigma). Proteins in gels were denatured, renatured, and assayed for kinase activity, as described by Suzuki and Shinshi (1995). Radioactivity in dried gels was analyzed and quantified using a Phosphorlimage (Molecular Dynamics, Sunnyvale, CA).

Induction of Ethylene Production in Tomato Leaf Pieces

Fully expanded leaves of 2- to 3-month-old tomato plants (L. esculentum cv Money maker) grown in the greenhouse were cut into 2-mm-thick slices and floated on water overnight. Slices were transferred to glass tubes (three slices corresponding to ~0.12 g fresh weight per 6-mL tube) containing 1 mL of an aqueous solution of the peptide being tested. After vacuum infiltration of the leaf slices (3 min with a water pump), vials were closed with rubber septa, and ethylene accumulating in the free air space was measured by gas chromatography after 2 hr.

Membrane Preparations and Binding Assays

Microsomal membranes were prepared from suspension-cultured cells of L. pereuvianum cells, as described previously (Grosskopf et al., 1990), except that cells were broken in a Parr cell disruption bomb (Parr Instrument Co., Moline, IL) rather than a French press. For binding assays, aliquots of microsomal membranes containing 200 μg of protein were incubated with 76 fmol syst-Tyr-I25 (>2000 Ci/mmol) in a total volume of 200 μL of binding buffer. Binding buffer was designed to minimize nonspecific binding and contained 25 mM Tris-Mes, pH 7.4, 10 mM MgCl2, 10 mM KI, 5 mM 3-iodo-L-tyrosine, 5 mM L-lysine, 0.1% trypotone, and 0.1% BSA. After incubation on ice for the times indicated, microsomal membranes were collected by vacuum filtration on glass fiber filters (model GF/B; Whatman) and washed for 5 sec with 15 mL of ice-cold washing buffer (25 mM Tris-Mes, pH 7.4, 100 mM MgCl2, 100 mM KCl, 10 mM KI, 0.1% trypotone, and 0.1% BSA). Radioactivity retained on the filter was measured by γ-counting.

Reproducibility

The results shown represent single experiments that are representative of at least three independent repetitions. In different batches of the cell cultures, the alkalization response varied somewhat with regard to the absolute pH increase (∆pHmax between 0.8 and 1.4) as well as with regard to the sensitivity of the cells (EC50 between 0.03 and 0.2 nM).

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