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*Le*RALF, a plant peptide that regulates root growth and development, specifically binds to 25 and 120 kDa cell surface membrane proteins of *Lycopersicon peruvianum*

Abstract A photoaffinity analog of tomato leaf RALF peptide (*Le*RALF), ^{125}I-azido-LeRALF, bound saturably to tomato suspension cultured cells in the dark in a classical receptor binding assay. Classical kinetic analyses revealed that the analog interacted with a single binding site on the surface of the cells with a K_D of 0.8×10^{-9} M, typical of known peptide hormone–receptor interactions in both plants and animals. The ^{125}I-azido-*Le*RALF, when exposed to UVB light in the presence of the cells, strongly labeled only two proteins of 25 kDa and 120 kDa, with the 25 kDa protein being more strongly labeled than the 120 kDa protein. The cell-surface localization of the interaction was indicated, as suramin, a known inhibitor of peptide–receptor interactions, and native *Le*RALF peptide competed with ^{125}I-azido-*Le*RALF labeling of both proteins. Two biologically inactive *Le*RALF analogs were not competitors. Incubation of ^{125}I-azido-*Le*RALF with suspension cultured cells in the dark, where it was fully active, could subsequently be totally dissociated from cells by acid washes, indicating that it was interacting at the cell surface and was not internalized. The ^{125}I-azido-*Le*RALF-labeled 25 kDa and 120 kDa proteins could not be solubilized from cell membranes by methods that release peripheral proteins, indicating that they are integral membrane components. The cumulative kinetic and biochemical evidence strongly indicates that the two proteins may be components of a *Le*RALF receptor complex.

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Introduction

Within the past decade, several peptide hormones have been discovered in plants that play important roles in diverse physiological processes, including inducible defense responses (Pearce et al. 1991, 2001a; Pearce and Ryan 2003), cell division and differentiation (Matsubayashi and Sakagami 1996), growth and development (Fletcher et al. 1999; Pearce et al. 2001b), and reproduction (Schopfer et al. 1999).

Of the plant peptide signals now known, receptors have been identified for only four. The systemin receptor (SR160)(Scheer and Ryan 2002), the PSK receptor (Matsubayashi et al. 2002) and the CLV3 receptor (CLV1) (Clark et al. 1997) are members of the LRR receptor kinase family, while the SCR receptor (SRK) (Kachroo et al. 2002) is a cysteine-rich receptor kinase. The CLV1 and SRK genes were identified by map based cloning, and the SR160 and PSK receptor genes were identified by first identifying the receptors with photoaffinity labels, and then purifying the proteins from membranes of suspension cultured cells and subsequently isolating the cDNAs using probes prepared from amino acid sequence data.

*Nt*RALF (*Nicotiana tabacum* rapid alkalinization factor) is a 49 amino acid peptide that was first identified by its ability to cause a rapid alkalinization response of the medium of tobacco suspension cultured cells and a rapid activation of a MAP kinase activity when added to the cells at nanomole concentrations (Pearce et al. 2001b). The alkalinization of suspension-cultured cells is a receptor-mediated response that results from the inhibition of a membrane-bound proton ATPase (Stratmann et al. 2000; Meindl et al. 1998), and has been a key assay for the isolation of several plant peptide hormones (Pearce et al. 2001a, b). Synthetic tomato *Le*RALF

peptide (*Lycopersicon esculentum* RALF), when supplied to solutions in which tomato and Arabidopsis seedlings are germinating, causes an immediate arrest of root growth (Pearce et al. 2001b). By transferring seedlings to fresh medium lacking the *Le*RALF peptide, growth arrest can be reversed. The rapid effects of very low concentrations of the *Le*RALF peptide (10^{-9} M) that cause the alkalinization response of cultured cells and inhibition of root growth and development suggested that the responses to *Le*RALF, like other animal and plant peptide hormones, are receptor mediated.

The *Le*RALF precursor cDNA (Pearce et al. 2001b) encodes a 115 amino acid precursor that exhibits three domains: a leader sequence of 23 amino acids; a highly conserved C-terminal domain of 50 amino acids (> 80% identity among RALF peptides from tobacco, tomato, and Arabidopsis) that constitutes the *Le*RALF peptide; and an upstream sequence of 43 amino acids between the leader sequence and the *Le*RALF sequence. *RALF-like* genes have been identified in 16 species of plants from nine families (Pearce et al. 2001b). A large family of putative *RALF-like* paralogs are present in the Arabidopsis genome whose putative C-terminal RALF peptide coding regions exhibit homology to those of tobacco (Olson et al. 2002) and the expression patterns of many of the genes are tissue specific (Pearce et al. 2001b). An Arabidopsis leaf-specific *RALF precursor* gene, fused with cDNA-green fluorescent protein (GFP) and expressed in leaves using a high-throughput viral expression system (Escobar et al. 2003), was first detected in the ER, then in cell walls, and was finally found only in the cell wall. Five RALF orthologs were isolated from fertilized ovule and ovary cDNA libraries of *Solanum chacoense* (*Sc*RALF peptides) that were predominately expressed in ovaries and large fruits (Germain et al. 2005), supporting the proposed roles for RALF peptides as signals involved in plant developmental processes.

The presence of leader sequences on all *At*RALF precursor proteins, together with the localization of the *At*RALF precursor protein in cell walls, indicates that the cell-wall-associated precursor may be processed extracellularly in response to a physiological cue(s), and may exert its biological activity through a specific interaction with a cell surface membrane receptor. Our identification of two cell surface *Le*RALF-binding proteins of tomato cells by specifically tagging them with a radioactive label provides an important first step toward the isolation and characterization of the *Le*RALF receptor protein and its gene.

Materials and methods

Plant cell cultures

Suspension cultured cells were maintained in MS media as previously described (Pearce et al. 2001a). The alkalinization assay was as previously described, using cells for assay 3–5 days after transfer. One milliliter of cells was placed into each well of 24-well culture plates (Phoenix, Hayward, CA, USA) and allowed to equilibrate while agitated on an orbital shaker (160 rpm) for 1 h. One to 10 µl of fractions to be assayed were added to the cells and the increase in pH of the cells was monitored.

Suramin inhibition of *Le*RALF biological activity

A stock solution of 10 mM suramin (Sigma, St. Louis, MO, USA) solution was prepared in water from which several dilutions were made. Ten microliters of each dilution was added to the cells 5 min prior to addition of either 10 µl of 250 nM tomato systemin (an 18 amino acid defense signaling peptide) or 250 nM *Le*RALF. Alkalinization of the media of tomato suspension cultured cells by each sample was recorded and expressed as change in pH/10 min. Incubation of cells with suramin for periods of over 10 min at the highest concentration added did not induce an alkalinization response (Stratmann et al. 2000).

Construction and analysis of azido-*Le*RALF

Biologically active synthetic *Le*RALF, with its two disulfide cross-links in the correct orientation, was prepared according to the methods described previously (Pearce et al. 2001b). All manipulations involving the azido containing SASD (a heterobifunctional cross-linker sulfosuccinimidyl-2[*p*-azido-salicylamido]ethyl-1,3′-dithiopropionate; Pierce, Rockford, IL, USA) were performed under red light. SASD was coupled to *Le*RALF through formation of an amide bond between the amine reactive sulfosuccinimidyl group of the crosslinker and primary amines of *Le*RALF. From a stock solution of 10 mM SASD in dimethylformamide, 20 µl were added slowly (5 µl at a time) to 100 µl of 0.1 M sodium phosphate buffer, pH 7.2. To this solution, 40 µl of 2.5 mM synthetic *Le*RALF (100 nmol) were added and the reaction was allowed to proceed for 2 h at room temperature. The reaction was quenched by the addition of 1 ml of 0.1% trifluoroacetic acid (TFA) and the products were purified on an analytical C18 RP-HPLC column (4.6 mm × 250 mm, Vydac, Hesperia, CA, USA) with a linear gradient of acetonitrile/0.1% TFA from 0 to 50% over 90 min. With a 1 ml/min flow rate, 1 ml fractions were collected and those containing either un-reacted *Le*RALF or azido-*Le*RALF adducts were identified by mass spectroscopy (cf. Fig. 2c) and their biological activities assayed adding 1 µl of each fraction to 1 ml of tomato suspension cultured cells and measuring the resulting medium alkalinization as previously described (Pearce 2001a, b). The peaks containing azido-*Le*RALF were lyophilized, re-suspended in 100 µl water, and chromatographed on a narrow bore C18 RP-HPLC column (2.1 mm × 250 mm, Vydac) with an

acetonitrile gradient of 25–40% (0.1% TFA) over 90 min with a 0.25 ml/min flow rate. Fractions of 0.25 ml were collected and assayed for biological activity in the alkalinization response as described above. Active fractions from the narrow bore separation were lyophilized and re-suspended in 100 μl water and the protein was quantified (BCA assay, Pierce, Rockford, IL, USA) using synthetic LeRALF as a standard. Mass spectral analyses of the fractions were performed on an LCQ mass spectrometer using electrospray ionization (ESI, Finnigan-Thermoquest, San Jose, CA, USA). Samples were infused into the mass spectrometer after diluting 1 μl (∼100 pmol) of the quantified sample in 50 μl of 3.0% formic acid. Positive ion mode was used for detection and mass/charge distributions were deconvoluted manually or by using BIOEXPLORE (Finnigan-Thermoquest).

The biological activities of azido-LeRALF fractions from the HPLC separations were quantified by assaying each fraction with tomato suspension cultured cells and monitoring the changes in pH after 10 min. The activities are reported as the effective concentrations that produce 50% of the activity of wild-type LeRALF peptide (EC_{50}).

Preparation of inactive alkylated and oxidized LeRALF derivatives

Alkylated LeRALF was prepared as described previously (Pearce et al. 2001b). A mixture of inactive LeRALF peptides (oxy (-) LeRALF), having the two disulfides in incorrect orientations, were isolated and assayed as described by Pearce et al. (2001b).

Iodination of azido-LeRALF

Radio-iodinated azido-LeRALF was prepared in the dark as follows: to 100 μl of 0.1 M sodium phosphate pH 7.5, 15 μl of azido-LeRALF (2.0 nmol), 2 mCi of [125I]Na containing 2,200 Ci/mmol (NEN/DuPont, Wilmington, DE, USA) were added. Iodination was initiated by the addition of 1 Iodobead (Pierce, Rockford, IL, USA) pre-rinsed in the phosphate buffer and the reaction was allowed to proceed. At 5 min, the solution was mixed by drawing the mixture into and out of a pipette, and then incubating for an additional 2 min. The iodinated azido-LeRALF (125I-azido-LeRALF) was purified by RP-HPLC as described above. Ten microliters of each fraction was assayed for radioactivity using an Isodata 2020 gamma counter, and the peak tubes of radioactivity were stored at 4°C in the dark. The specific activity of 125I-azido-LeRALF was 2,200 Ci/mmol. Iodinated azido-LeRALF exhibited the same alkalinization activity as LeRALF or azido-LeRALF (cf. Fig. 3), indicating that the presence of the iodine on the azido-LeRALF did not affect its biological activity (data not shown).

Receptor binding and photoaffinity labeling assays

Tomato suspension cells, 4–8 days after sub-culturing, were employed for alkalinization assays, for receptor binding and for photoaffinity labeling assays. For receptor binding assays without activation of the azido cross-linker, 1 ml aliquots of suspension-cultured cells in 24-well culture plates were incubated in the dark on an orbital shaker at ∼180 rpm. Prior to addition of 125I-azido-LeRALF, 40 μl of 1 M sodium acetate buffer, pH 5.5 and 0.02 mg BSA were added to the cells. 125I-azido-LeRALF (1×10^6 cpm) was added to the cells in the culture plates and incubated for 5 min, unless otherwise indicated. After incubation, cells were filtered on two layers of Whatman no. 2 filter paper on a 12-well vacuum filtration manifold and immediately washed with 5 ml of 4°C media. Radioactivity associated with the cells was determined by gamma counting. Total binding was determined by measuring radioactivity associated with the cells in the absence of unlabeled LeRALF and non-specific binding was determined by measuring the radioactivity associated with the cells in the presence of competing concentrations of unlabeled LeRALF (200 nM). Specific binding is expressed as the difference between total binding and non-specific binding, while the K_d and B_{max} were determined by Scatchard analysis of the saturation data.

Photoaffinity labeling of the proteins associated with LeRALF binding was performed under similar conditions. However, after filtering and washing the cells, the azido cross-linker was activated by irradiation with a UV-B lamp 6 cm above the filtration manifold (Scheer and Ryan 2002). After irradiation, cells were collected in Eppendorf tubes containing 250 μl extraction buffer (1 M sodium acetate, pH 5.5) and 10 μl of plant protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Crude extractions were made on ice by sonication twice for 10 s at 30% power (micro probe) using a Virsonic 475 Ultrasonic Cell Disrupter (VirTis Co., Gardiner, NY, USA). The extract was centrifuged for 5 min at 10,500 g (4°C) and 40 μl of the soluble extract was added to 10 μl of non-reducing SDS-PAGE sample buffer. The samples were boiled for 5 min and separated by 10% SDS-PAGE using a mini-Protean II gel system (Bio-Rad, Hercules, CA, USA). The gels were stained with Coomassie brilliant blue (CBB), dried between cellophane and analyzed for radioactivity by phosphor imaging.

Characterization of 125I-azido-LeRALF labeled proteins

For competitive binding assays, LeRALF was dissolved in water and added to 1 ml of suspension-cultured cells immediately prior to the addition of 125I-azido-LeRALF, and photoaffinity reaction was carried out as described above. Alkylated LeRALF (in which all half cystines were blocked) and oxi(-) LeRALF (which contained incorrect disulfide bond orientations and were

biologically inactive), were assayed for their competition for labeling of the cells by ^{125}I-azido-LeRALF.

The saturation of the binding of ^{125}I-azido-LeRALF to suspension cultured cells was determined by adding increasing amounts of ^{125}I-azido-LeRALF to 1 ml of cells in the presence or absence of 200 nM competing LeRALF. The labeling of the proteins was assessed by SDS-PAGE/phosphor imaging.

Membranes were isolated from the cells to determine if the photoaffinity-labeled proteins were membrane bound. Cells containing the ^{125}I-azido-labeled proteins were extracted as previously described (Scheer and Ryan 2002) and membranes were isolated from soluble proteins by centrifuging the crude extracts at 38,000 g for 45 min in an F-20 microrotor at 4°C. The membrane pellets were re-suspended in 100 μl 10% SDS and assayed by SDS-PAGE as described above. To determine if the labeled proteins were integral or peripheral membrane proteins, ^{125}I-azido-labeled membranes were isolated and resuspended in 125 μl of water and 25 μl of the suspended membranes were aliquoted into five Eppendorf tubes to which 450 μl of each of the following washing solutions were added: (1) water, (2) 1.5 M NaCl, Tris–EDTA (50, 1.0 mM) pH 8.8, (3) 1.5 M NaCl, 1% formic acid pH 2.0, (4) 1.0 M borate buffer pH 10.0, or (5) 8 M urea, pH 8.5. Each of the samples were vortexed for 30 s, incubated on ice for 30 min, and the membranes pelleted by centrifugation at 38,000 g for 30 min. The membrane pellets were dispersed by brief vortexing in 1 ml of water and re-centrifuged for 15 min to remove excess solvents. The membrane-associated proteins remaining were separated by SDS-PAGE and analyzed by phosphor imaging.

Results

To investigate the mechanism of perception of $RALF$ by plant cells, the presence of receptor proteins or other binding proteins on the cell surface of membranes of tomato suspension cultures was sought. Initially we assayed the effects of suramin, a heterocyclic, polysulfonated inhibitor of ligand–receptor interactions in both animals and plants (see references in Stratmann et al. 2000), for its ability to inhibit the alkalinization response in tomato cells induced by LeRALF. Suramin strongly inhibited LeRALF-induced alkalinization of tomato suspension cell culture medium (Fig. 1), exhibiting an IC_{50} of about 20 μM, which is equal to or less than the IC_{50} recorded for inhibiting receptor function in animals and plants, supporting the hypothesis that the biological activity of RALF peptides may be receptor mediated (Pearce et al. 2001b).

A photoaffinity analog of LeRALF was synthesized by coupling SASD, an amine reactive heterobifunctional photocross-linker, to synthetic LeRALF. The azido-LeRALF products were separated on an analytical C18 RP-HPLC column (Fig. 2a) and the fractions were collected and assayed for biological activity. Unreacted
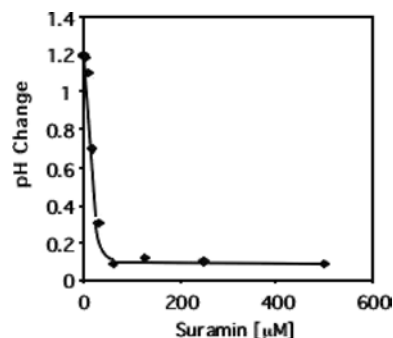

Fig. 1 Suramin inhibits RALF-induced medium alkalinization of tomato suspension cultured cells. Suramin was added to 1 ml aliquots of suspension cultured tomato cells in 24-well culture plates and shaken at 160 rpm on an orbital shaker. After a 5 min incubation time, 2.5 pmol of LeRALF was added to the cells and the change in pH of the suspension cell culture medium was recorded 10 min later

LeRALF eluted in fraction 48, and an overlapping mixture of active peaks eluted in fractions 60 and 61. Fractions 60–61 were pooled and the products further separated using a narrow bore C18 RP-HPLC column (Fig. 2b). Three defined peaks were recovered, each having biological activity in the alkalinization assay using tomato suspension cultured cells. All three peaks of the triad were analyzed by mass spectrometry and each exhibited an identical mass corresponding to a singly modified LeRALF, as shown in Fig. 2c. The three active peaks were pooled and termed azido-LeRALF. The biological activity of azido-LeRALF was determined to be similar to native LeRALF, with an EC_{50} of about 5 nM (Fig. 2d).

Iodination of ∼5.0 nmol azido-LeRALF with 2 mCi Na^{125}I produced a ^{125}I-azido-LeRALF that, when purified by RP-HPLC, had a later retention time than azido-LeRALF. One major peak of radioactivity resulted that exhibited a specific activity of 2,200 Ci/mmol. This ^{125}I-azido-LeRALF was used in all subsequent photoaffinity-labeling experiments.

Initially, the presence of a ^{125}I-azido-LeRALF binding site was identified without activation of the photoaffinity cross-linker (Fig. 3a). Incubation of suspension cultured cells with increasing concentrations of ^{125}I-azido-LeRALF followed by determination of the specific binding indicated that a single class of high affinity binding sites was saturated by ^{125}I-azido-LeRALF at about 2 nM. The affinity of the sites was estimated by a Scatchard plot to be 0.8 nM with a population of 135 fmol/10^6 cells (Fig. 3b). Incubation of ^{125}I-azido-LeRALF with tomato suspension cultured cells for 5 min followed by UV irradiation and SDS-PAGE/phosphor imaging resulted in the detection of two strongly labeled proteins with apparent MWs of 25 kDa and 120 kDa and a weakly labeled, high molecular weight protein (Fig. 3c). Photoaffinity labeling showed a saturation of all three proteins at about 2 nM, similar to that of whole cells (Fig. 3a). The azido-labeling of the proteins was competed by unlabeled LeRALF at
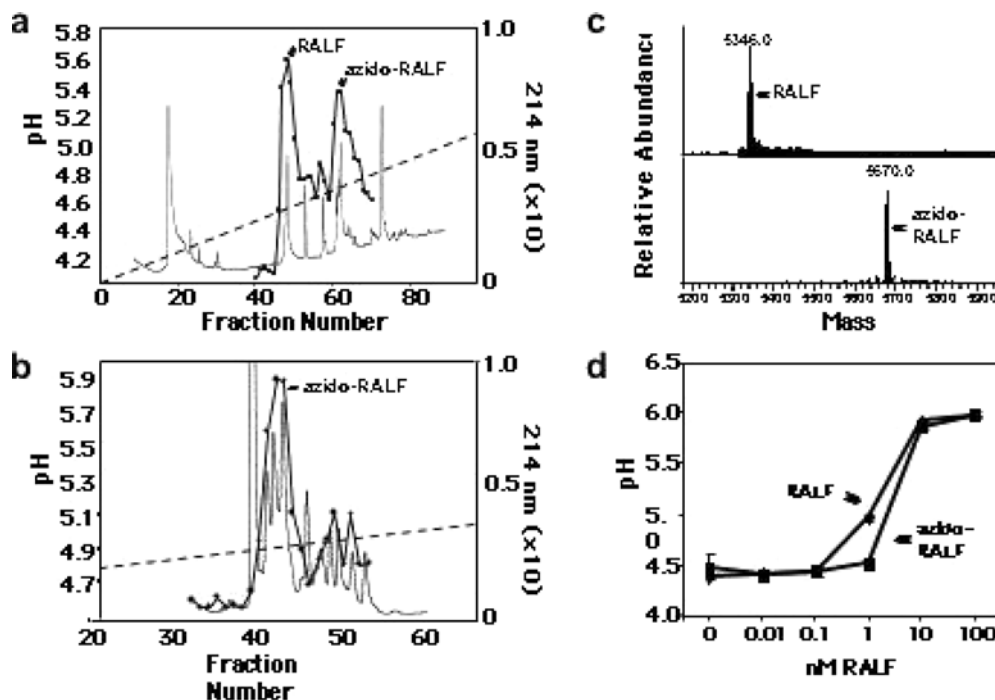
Fig. 2 Isolation of azido-RALF. **a** Synthetic *Le*RALF peptide was coupled to the photoaffinity cross-linker SASD and the products were purified by C18 RP-HPLC with a linear gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid (TFA) over 90 min (*dashed line*). UV absorbance of the eluted reaction products was monitored at 214 nm (*solid line*). Biological activity of 1 μl of each fraction was assayed by adding to 1 ml of tomato suspension cultured cells and measuring the resulting increase in pH of the medium after 10 min as in Fig. 1 (*solid line with dots*). **b** The fractions from **a** containing biologically active azido-RALF were pooled, lyophilized, re-suspended in water, and chromatographed on a narrow bore C18 RP-HPLC column with a 25–40% gradient of acetonitrile in 0.1% TFA over 90 min (*dashed line*). UV absorbance was determined at 214 nm (*solid line*) and 1 μl of each fraction was assayed for biological activity as in Fig. 1 (*solid line with dots*). **c** The mass of each of the three azido-RALF peaks in **b** was determined by electrospray mass spectrometry. A representative mass spectrum of azido-RALF species (*lower panel*) is compared to the mass spectrum of the native RALF peptide (*upper panel*). Each of the three peaks exhibited the same molecular mass. **d** The biological activity of native RALF and azido-RALF was assayed as in Fig. 1. The pH of the cell culture medium in each well was monitored and the change after 10 min is shown. *Error bars* indicate the standard deviation of three experiments

concentrations as low as 2 nM, and was completely blocked between 2 nM and 20 nM (Fig. 4). Competition with biologically inactive reduced and alkylated *Le*RALF (alk *Le*RALF), and a mixture of *Le*RALF species having incorrect disulfide bonds (oxi(-) *Le*RALF) (Pearce et al. 2001b), did not compete with labeling of the proteins by ^{125}I-azido-*Le*RALF (Fig. 4, right lanes).

The labeled proteins were found to be associated only with the membrane pellet (Fig. 5a). Treatment of the membranes with β-Me did not result in a molecular weight shift of the 25 kDa or 120 kDa proteins, but the weakly labeled higher molecular weight protein disappeared. This indicated that neither the 25 kDa or 120 kDa protein were composed of smaller polypeptides

linked by disulfide bridges, but that the higher molecular weight protein may be a minor aggregate composed of the 25 kDa and 120 kDa proteins. Agents known to solubilize peripheral proteins from membranes such as high salt or extreme acid or base did not solubilize either the 25 kDa or 120 kDa proteins, but the acid treatment caused the disappearance of the weakly labeled larger component (Fig. 5b). As expected, treatment of membranes with 8 M urea partially solubilized the radioactivity of both proteins in membranes and reduced the total background radioactivity. The degree of CBB staining of membranes was also reduced by the urea treatment, indicating the solubilization of most membrane proteins (Fig. 5b).

The presence of ^{125}I-azido-*Le*RALF binding proteins was examined in suspension-cultured cells from tobacco and alfalfa, which respond to *Le*RALF with a strong medium alkalinization (G. Pearce and C. A. Ryan, unpublished data). The 120 kDa protein was present in both species, but the 25 kDa protein was not (Fig. 6). Instead, tobacco exhibited labeling of a 35 kDa protein and alfalfa only a weak, diffuse smear present at about 45 kDa. The weakly labeled higher molecular weight band was present near the origin of the lanes in Figs. 4 and 6 and appeared to disappear in the presence of unlabeled *Le*RALF.

Discussion

Peptide hormones were only recently identified in plants as important signaling molecules (Pearce et al. 1991), and about a dozen plant peptide signals have now been reported. Many of the plant peptide signals have similarities to animal peptide hormones such as being active
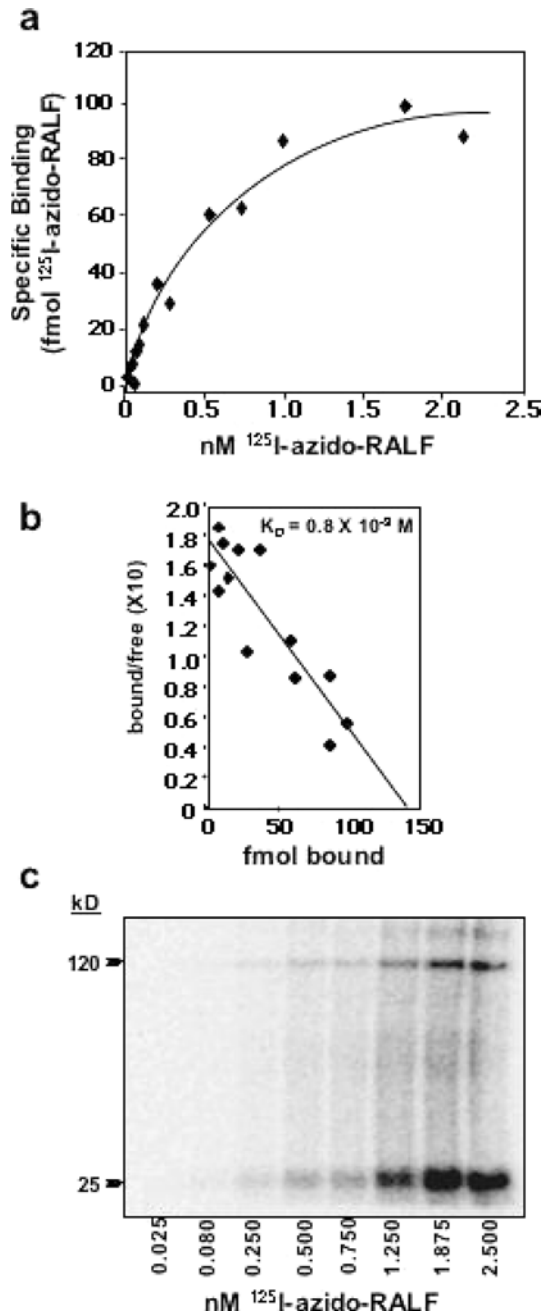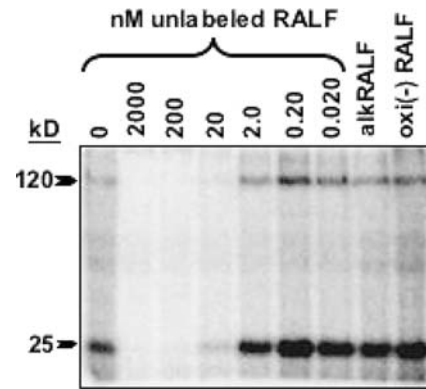
Fig. 4 Electrophoretic analysis of the photoaffinity labeling of tomato suspension cultured cells in the presence of competing concentrations of native RALF peptide. Tomato suspension cultured cells were aliquoted (1 ml) into 24-well culture plates and incubated on an orbital shaker (160 rpm). The cells were treated with 1×10⁶ cpm of ¹²⁵I-azido-RALF and agitated in the dark for 5 min, filtered, washed with 5 ml buffer at 4°C, and irradiated with UV light. Cells were broken by ultrasonication and the extracted proteins were analyzed by SDS-PAGE and phosphor imaging (*lane 1*). Identical experiments were performed with the addition of increasing concentrations of unlabeled synthetic RALF (*middle lanes*), with 200 pmol of reduced/alkylated RALF (*alk-RALF*), or RALF with non-native disulfide bridges (*oxi(-)RALF*) immediately prior to the addition of 1.0×10⁶ cpm ¹²⁵I-azido-RALF

Fig. 3 Saturable binding to suspension cultured cells and labeling of the 120 kDa and 25 kDa proteins with increasing concentrations of ¹²⁵I-azido-RALF. **a** Tomato suspension cultured cells in 1 ml aliquots were incubated in the dark with increasing concentrations of ¹²⁵I-azido-RALF in the presence (non-specific binding) or absence (total binding) of competing concentrations of unlabeled RALF. After 5 min the cells were collected without irradiation by filtration and washed with 5 ml chilled buffer. The radioactivity associated with the cells was determined by a gamma counter and expressed as specific binding (total binding minus non-specific binding). **b** Scatchard plot of the data from **a**. **c** Tomato suspension cells in 1 ml aliquots were incubated in the dark with increasing concentrations of ¹²⁵I-azido-RALF. After 5 min the cells were collected by filtration, washed with 5 ml chilled buffer, and irradiated with UV light. Proteins labeled by the photo-affinity reagent were determined by SDS-PAGE followed by phosphor imaging
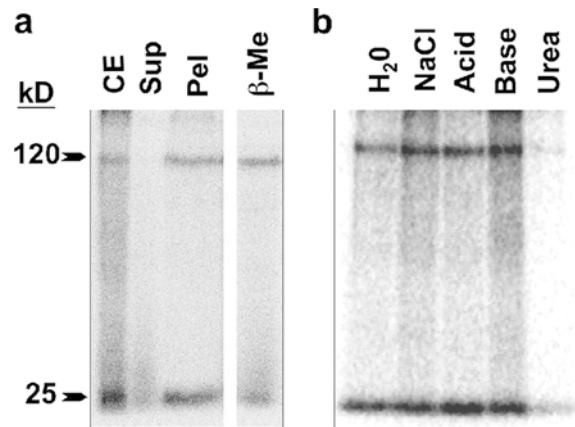
Fig. 5 The 25 kDa and 120 kDa ¹²⁵I-azido-RALF-labeled proteins behave as integral cell membrane proteins. **a** One ml of tomato suspension cultured cells were labeled using 1.0×10⁶ cpm of ¹²⁵I-azido-RALF, disrupted by ultrasonication, and the entire cell extract was analyzed by SDS-PAGE and phosphor imaging (*lane 1*). Membranes and soluble proteins were separated by centrifugation and the supernatant (*lane 2*) and pellet (*lane 3*) were each analyzed by SDS-PAGE and phosphor imaging. The presence of intermolecular disulfide bridges was investigated by boiling the membranes in 5% β-Me followed by SDS-PAGE and phosphor imaging (*lane 4*). *CE* Crude extract, *Sup* supernatant, *Pel* pellet, *β-Me* β-mercaptoethanol. **b** Membranes were isolated from tomato suspension cells that were labeled with ¹²⁵I-azido-RALF as in **a**. Aliquots of the membranes were incubated under various conditions for 30 min to dissociate peripheral membrane proteins. Membranes were then collected by centrifugation, and radiolabeled proteins that bound to the membranes were analyzed by SDS-PAGE and phosphor imaging. *NaCl* 1.0 M sodium chloride in Tris–EDTA, pH 8.0; *Acid* 3.0% formic acid; *Base* 1.0 M sodium borate, pH 10.0; *Urea* 8 M urea, pH 8.5
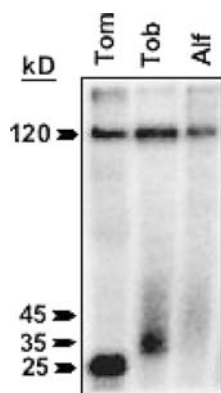
Fig. 6 ^{125}I-azido-RALF-labeling of cell surface proteins of suspension cultured tobacco and alfalfa cells. One-milliliter suspension cultures of tomato, tobacco and alfalfa were photoaffinity labeled with 1.0×10^6 cpm of ^{125}I-azido-RALF as described in Fig. 2. The cells were disrupted by ultrasonication and the proteins analyzed by SDS-PAGE and phosphor imaging

at fmol levels, being cleaved from prohormone precursors, and being perceived by specific receptors on the surface of target cells (Ryan et al. 2002). Several of the peptides cause an alkalinization of suspension cultured cells, due to the interaction of the receptors with intracellular signaling that blocks a membrane bound ATP driven proton pump (Meindl et al. 1998; Schaller and Oecking 1999). Fundamental to understanding mechanisms of peptide perception and signal transduction is the identification, isolation and characterization of the various receptors. Only four plant peptide hormone receptors have been identified and characterized, and the *Le*RALF peptide provided the opportunity to seek its receptor on suspension cultured tomato cells and to eventually clone the gene and characterize its regulation and function.

The possibility that the biological acivity of *Le*RALF was receptor-mediated was strengthened by the finding that suramin, a potent inhibitor of receptor–ligand interactions (Stratmann et al. 2000), was found to block the alkalinization response caused by *Le*RALF in tomato suspension cultured cells (Fig. 1). While the mechanism of suramin inhibition is not fully understood, it powerfully interacts with membrane receptors to block ligand binding in a non-specific manner.

The coupling of the photoaffinity reagent SASD with amino groups in *Le*RALF to form azido-*Le*RALF resulted in a mixture of azido-*Le*RALF species that were separated by HPLC and assayed (Fig. 2a). Analogs that potently induced medium alkalinization of tomato suspension cultured cells (Fig. 2a) were further purified (Fig. 2b) and were found to exhibit identical molecular weights of 5,670, determined by mass spectrometry (Fig. 2c). This mass corresponded to native *Le*RALF that had been modified with one cross-linker. However, because of their different elution characteristics, it is likely that the three species are labeled with a single cross-linker at different amino acid residues in each of

the peptides. There are five possible attachment sites in the peptide, the N-terminus and four lysines at positions 3, 4, 13, and 29. The retention of nearly full biological activity of azido-*Le*RALF triad (Fig. 2d) indicated that the single modifications, resulting in a reduction of the net positive charge of each *Le*RALF species, did not diminish their biological activities, nor did iodination of the pooled azido-*Le*RALF peaks (data not shown). The three fractions were pooled and called ^{125}I-azido-*Le*R-ALF, which was employed for all of the photoaffinity labeling experiments reported herein.

Binding of ^{125}I-azido-*Le*RALF to cells in the dark (to avoid cross linking) exhibited a steady-state saturation with a K_d that is indicative of a single high affinity binding site (Fig. 3 a, b). Photoaffinity labeling of the whole cells identified 25 kDa and 120 kDa proteins present in the cell membranes that exhibited saturation near 2 nM (Fig. 3a, c), although the radioactivity incorporated into the 25 kDa protein was much higher than that of the 120 kDa protein (Fig. 3c).

Competition analyses between synthetic *Le*RALF peptide and ^{125}I-azido-*Le*RALF indicated that a concentration of 20 nM *Le*RALF strongly blocked labeling of both the 25 kDa and 120 kDa proteins by ^{125}I-azido-*Le*RALF (Fig. 4). On the other hand, reduced/alkylated *Le*RALF (alk *Le*RALF) and *Le*RALF having improperly cross-linked disulfide bonds (oxi(-) *Le*RALF) did not compete with labeling (Fig. 4), supporting the high specificity of both *Le*RALF binding proteins. The correctly positioned disulfide cross-links in the RALF structure had been previously shown to be crucial for its biological activity (Pearce et al. 2001b), and are shown here to be critical for receptor binding.

The 25 kDa and 120 kDa proteins behave as integral membrane proteins, as treatments with salts or pH extremes did not solubilize either protein, nor did treatments designed to remove peripheral proteins, or other proteins loosely associated with the membrane (Fig. 5a, b). The possibility that the two proteins were proteolytically derived from a larger protein is not likely, since all experiments were performed in the presence of inhibitors of plant proteases and the stoichiometry of the levels of the two proteins has been constant throughout the entire study.

*Le*RALF is as active in inducing the alkalinization response in suspension-cultured cells of tobacco and alfalfa as RALF peptides that are native to these species (Pearce et al. 2001b). Labeling of tobacco and alfalfa suspension cultured cells with ^{125}I-azido-*Le*RALF in the identical manner as done with tomato cells indicated that a 120 kDa *Le*RALF-binding protein was present in each species (Fig. 6). However, a 25 kDa protein was not labeled in tobacco or alfalfa cell membranes. Instead, a protein of about 35 kDa was labeled in tobacco cells and a diffuse smear of about 45 kDa MW was labeled in alfalfa cells. It is not known if these proteins are related to the 25 kDa protein from tomato cells, or if the 25 kDa *Le*RALF binding proteins exists in tobacco

and alfalfa and was not labeled. The smaller labeled proteins may be ancillary components of a *Le*RALF receptor complex to which binding of RALF is essential for the initiation of biological activity.

The binding of ^{125}I-azido-*Le*RALF to cell surface membranes is supported by several lines of evidence. Binding is blocked by suramin, an inhibitor of peptide hormone-receptor interactions which does not penetrate the plasma membrane; the photoactive ^{125}I-azido-*Le*RALF label that is strongly bound to cells in the dark was released by acid washes, indicating that it was not internalized; Washee designed to remove peripheral proteins from the cells did not affect photoaffinty labeling; and the extraordinary specificity of the labeling of only two proteins with virtually no background labeling (Fig. 3c) is strongly competed by native *Le*RALF (Fig. 4) but not by biologically inactive *Le*RALF analogs. The cumulative data provides compelling evidence that the site of a LeRALF-receptor interaction is on cell surface membranes, and strongly supports a role for the 25 kDa and 120 kDa proteins as components of a *Le*RALF-receptor interaction linked to an intracellular signaling pathway.

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