A lipid transfer protein binds to a receptor involved in the control of plant defence responses

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Abstract Lipid transfer proteins (LTPs) and elicitins are both able to load and transfer lipidic molecules and share some structural and functional properties. While elicitins are known as elicitors of plant defence mechanisms, the biological function of LTP is still an enigma. We show that a wheat LTP1 binds with high affinity sites. Binding and in vivo competition experiments point out that these binding sites are common to LTP1 and elicitins and confirm that they are the biological receptors of elicitins. A mathematical analysis suggests that these receptors could be represented by an allosteric model corresponding to an oligomeric structure with four identical subunits.

Key words: Allostery; Elicitin; Lipid transfer protein; Receptor

1. Introduction

In the global concept of interactions between organisms and their environment, a major concern is to discriminate recognition between exogenous and endogenous signals, notably during pathogenic or allergenic interactions involving small proteins, such as lipid transfer proteins (LTPs) or elicitins.

LTPs are ubiquitous plant proteins able to load and transfer hydrophobic molecules such as fatty acids or phospholipids. Among them, LTPs 1 (type 1 LTPs) constitute a multigenic family of secreted plant lipid binding proteins that are constitutively expressed in specific tissues and/or induced in response to biotic and abiotic stress (for reviews see [1–4]). LTPs 1 are 9 kDa proteins, with an α-helix fold stabilized by four disulfide bonds, enclosing a hydrophobic tunnel with a unique plasticity enabling the binding of a large variety of lipids and hydrophobic molecules [1,2,5]. Their biological function is still unknown, even if some data provide arguments for a role of these proteins in the assembly of extracellular hydrophobic polymers (i.e. cutin and suberin) [2,4] and/or in plant defence against fungal pathogens [1,3]. Besides their involvement in plant defence, LTPs 1 are also known to be pan-allergens of plant-derived foods [6], as with other pathogenesis-related proteins [7].

Elicitins are (~10 kDa) monomeric proteins secreted by fungal pathogen from the *Phytophthora* or *Pythium* genera (Oomycetes) [8]. They display an α-helix fold stabilized by three disulfide bonds, which provides a hydrophobic cavity able to lodge sterol [9]. They are sterol carrier proteins [10] that afford capture of plant sterol necessary for the reproduction of these pathogens [11]. These proteins are also capable of binding phospholipids and fatty acids [12]. In plants, elicitins trigger a hypersensitive response which is associated with the induction of non-specific systemic resistance [13]. Related early steps in the signaling pathways involve the specific recognition of elicitins by high affinity plasma membrane proteins, a calcium signal, several protein phosphorylation steps and changes in membrane permeability to ions (H^+, K^+, Cl^-) leading to the production of active oxygen species (for review see [8]).

Finally, elicitins and LTPs 1 are secreted in the extracellular space and interfere with the plant cell wall organization [14,15]. They share some structural and non-specific lipid binding properties, but LTPs 1 are unable to bind sterols. Therefore, an obvious question rises: are LTPs 1 involved in the signaling pathways leading to the hypersensitive response triggered by elicitins? In order to address this question, we compared the binding properties of cryptogein, an elicitin secreted by *Phytophthora cryptogea*, and that of an LTP1 isolated from wheat, using purified tobacco plasma membranes.

2. Materials and methods

2.1. Tobacco cell treatments and biological assays

Cryptogein was purified according to [16]. Wheat LTP1 was purified from *Triticum aestivum* seeds as previously described [17]. Tobacco cells were prepared and used for determination of elicitin activities as previously reported [18,19]. Cells from cultures in exponential phase growth were collected by filtration, washed, and resuspended (0.1 g fresh weight/ml) in 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM

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K_2SO_4 and 2 mM Mes buffer adjusted to pH 5.75 with KOH. After a 2-h equilibration, tobacco cells were simultaneously treated with cryptogein (2 nM) and LTP1 (2–50 nM). The amount of induced active oxygen species was determined by chemiluminescence [19].

2.2. Binding and ligand replacement experiments, using ^{125}I-labeled proteins

Iodination of cryptogein or LTP1 was performed as previously described [20]. Specific radioactivity of labeled ligand was about 200 Ci/mmol. Plasma membrane-enriched fractions were obtained as previously reported [21]. Binding experiments and ligand replacement experiments were carried out as already reported [22]. Plasma membrane preparations containing about 50 μg protein were suspended in a final volume of 100 μl with binding buffer (25 mM Tris–Mes pH 7.0, 5 mM $MgCl_2$, 0.1 M sucrose and 0.1% bovine serum albumin (BSA)) and preincubated on ice for 30 min. Binding of [^{125}I]elicitin or of [^{125}I]LTP1 was carried out for 90 min on ice. Non-specific binding was determined in the presence of 10 μM unlabeled cryptogein or LTP1. Binding assays were stopped by rapid filtration under vacuum through GF/B glass-fiber filters (Whatman) presoaked for 60 min in 1% BSA. Then, the filters were immediately washed three times with 5 ml of ice-cold binding buffer, and the radioactivity remaining on filters was measured. The specific binding was calculated by subtracting the non-specific binding from the total binding.

Binding theoretical curves were calculated using the model described by Monod, Wyman and Changeux [23]. The binding function for a ligand can be written as: $B(u)/S = u/K_B(1+u/K_B)^{n-1}/(T+(1+u/K_B)^n)$, where n is the number of subunits, A and B are two accessible states for binding protein, T is the equilibrium constant for the A → B transition ($A = TB$), K_B is the microscopic dissociation constant for the ligand bound to a stereospecific site in B state and S is the maximum value of the binding when the ligand concentration (u) is very high. We obtained the optimal coefficients (T, S and K_B) of the above equation using a three step computation algorithm. The first step involves linear–log regression and gives rough estimates of K_B and T. The second improves the estimate of T. The third step uses a numerical iterative method, and allows to determine the values for T, S and K_B which minimize the error function E

$$E(K_B, T, S, k) = \frac{1}{Sk}\sqrt{\sum_{i=1}^{k}(B(u_i) - B_i^*)^2}$$

where B_i^* are the measured values, u_i the ligand concentrations and k the number of measures.

3. Results

3.1. Binding of [^{125}I]LTP1 and [^{125}I]cryptogein with tobacco plasma membranes

Cryptogein and wheat LTP1 bind to plasma membranes and specific binding was determined from the measures of total and non-specific bindings, as shown for wheat LTP1 (Fig. 1, inset). The saturability level of these specific binding sites is similar for both proteins (Fig. 1). However, 50% saturability was obtained at about 4 nM and 8 nM for cryptogein and wheat LTP1, respectively. In addition, although the cryptogein binding curves could be analyzed as hyperbolic curves [22,24,25], that of wheat LTP1 presented a sigmoidal shape. This shape suggests that the LTP binding site is oligomeric, the molecular interaction involving positive cooperativity. This could correspond to an allosteric model with a transition from an A conformer, with little or no affinity for the ligand, to a B conformer exhibiting high affinity for the ligand. This model was used to describe the interaction of LTP1 and plasmalemma binding sites. In order to determine binding parameters, we tested Monod et al.'s model [23]. Using a three step computation algorithm, we obtained a minimal value for the error function ($E = 3\times10^{-3}$) for $n = 4$ with an apparent dissociation constant K_B of 1.6 nM, an allosteric

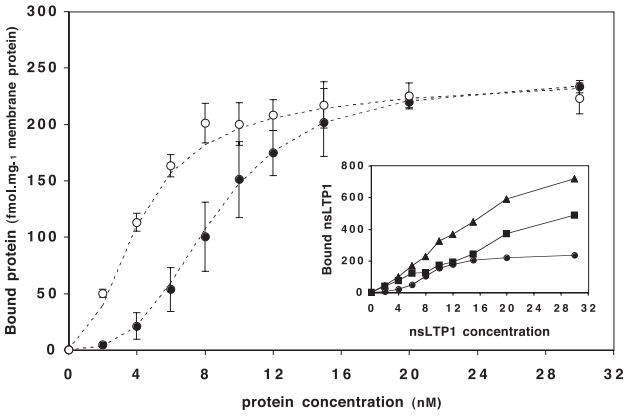

Fig. 1. Binding of LTP1 and cryptogein to tobacco plasma membranes. ^{125}I-labeled protein (LTP1 ● or cryptogein ○) was incubated with tobacco plasma membranes as previously described for cryptogein [22]. Inset represents the total (▲), the non-specific (■) and the specific binding (●) of LTP1. Dot lines represent the theoretical curves ($n = 4$) derived from the model described by Monod, Wyman and Changeux [23]. Experiments were repeated three times and results are the mean values ± S.D. (fmol of bound protein/mg plasmalemma protein).

constant T of 1252 and a saturation S of 248 fmol/mg membrane proteins. For these parameter values, the corresponding theoretical curve fits very well with the measured values (Fig. 1) whereas it did not either for $n = 2$ or $n = 6$, for which the minimal values of the error function are 20×10^{-3} and 7×10^{-3}, respectively. The addition of another parameter in the curve equation, which would take into account the binding of LTP1 to the A conformer, decreased the fitting to experimental values showing that the affinity of the ligand for the A conformer is negligible.

This model was also applied to the experimental data obtained with cryptogein. The calculation led to the following parameters: $n = 4$, $S = 246$ fmol/mg membrane proteins, $K_B = 2.0$ nM and $T = 30$. The corresponding theoretical curve fits very well with experimental values (Fig. 1). Here too, the fitting obtained for $n = 4$ ($E = 8\times10^{-3}$) is better than that for $n = 2$ and $n = 6$ ($E = 10\times10^{-3}$ and 12×10^{-3}, respectively). The cryptogein binding curves were previously deemed to be derived from a hyperbola-like relation [22,24]; but such a hyperbolic model failed to fit with experimental values, since its error function value ($E = 20\times10^{-3}$) is much higher than the one obtained with the allosteric relation.

Thus, the model with four subunits and a number of saturation sites of 247 ± 1 fmol/mg membrane proteins fits well with the experimental values for both, cryptogein and LTP1. The apparent binding constants K_B are very similar but the allosteric constants T are very different showing that cryptogein is more efficient than LTP in changing the binding protein conformation towards the B conformer. These results point out that cryptogein and LTP1 bind to high affinity specific sites located on the plasma membranes of tobacco and that the saturation level of these sites is similar for both proteins.

3.2. Displacement experiments

Displacement experiments were performed in order to determine if LTP1 and cryptogein share the same binding sites. Tobacco plasma membranes were preincubated with ^{125}I-la-

beled LTP1, before the addition of unlabeled LTP1, cryptogein, or lysozyme as a negative control. Both LTP1 and cryptogein were able to displace labeled LTP1, whereas lysozyme was not. The displacement kinetics were similar, with radioactivity associated with the membranes decreasing from 100 to 30 or 25% within 45 min following the addition of unlabeled LTP1 or cryptogein, respectively (Fig. 2). Similarly, bound [^{125}I]cryptogein could be displaced by unlabeled cryptogein or LTP1 (Fig. 2, inset). Thus, this experiment demonstrates that the specific binding sites for LTP1 and cryptogein are identical, and that the LTP1 interaction with the binding sites is reversible, as for cryptogein [22]. It can be concluded that the LTP1 binding sites exhibit all the characteristics of putative receptors.

3.3. In vivo competition

Finally, the effects of LTP1 on tobacco cells were analyzed. Even at 100 nM, LTP1 did not trigger the classical responses induced by 2 nM cryptogein on tobacco cell suspensions, such as extracellular medium alkalization or oxidative burst. Then, we investigated if the binding of wheat LTP1 could modulate the oxidative burst induced by cryptogein, when both proteins were simultaneously added to a tobacco cell suspension. The addition of increasing concentrations of LTP1 reduced the production of active oxygen species, induced by a fixed cryptogein concentration. This production was 50% inhibited at equimolar concentrations of the two proteins and was abolished with a two-fold excess of LTP1. Added at higher concentrations (up to 50 nM), LTP1 did not induce any additional changes (Fig. 3). This result indicates that the binding sites of LTP1 and cryptogein are truly their biological receptors. The difference we observed in protein biological activity can be explained by their efficiency to induce the conformational changes of the receptor subunits (*T*). LTPs 1 could also act as elicitin antagonists.

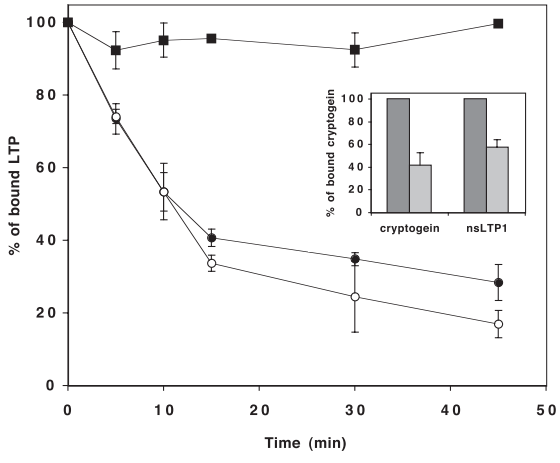

Fig. 2. Reversibility of wheat LTP1 or cryptogein specific binding to tobacco plasma membranes. Kinetics of displacement of [^{125}I]LTP1 bound to plasma membrane by unlabeled LTP1 ●, cryptogein ○ and lysozyme ■. Inset: displacement of [^{125}I]cryptogein specifically bound to plasma membrane by unlabeled cryptogein or LTP1, 10 min after the addition of the unlabeled protein. The experiments were repeated three times and results are the mean values ± S.D.

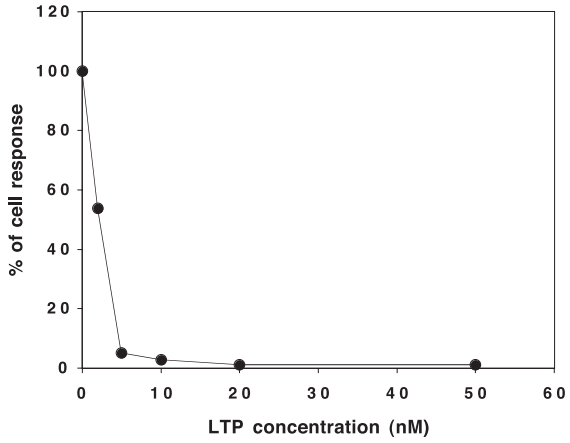

Fig. 3. In vivo competition between a wheat LTP1 and cryptogein. Tobacco cells were simultaneously treated with cryptogein (2 nM) and LTP1 (2–50 nM). Results are expressed in percentage of H_2O_2 produced by tobacco cells in the presence of cryptogein (2 nM), without LTP.

4. Discussion

The results reported here show for the first time that LTPs interact with receptors located on plant plasma membranes. These receptors have previously been identified as elicitin receptors [26]. Analysis of the cryptogein and wheat LTP1 binding curves indicates that receptors could be represented by a molecular model with an oligomeric structure, involving four identical subunits with a symmetric quaternary geometry. This is in accordance with a previous hypothesis, based on biological data [26]. However, although the formation of a sterol–elicitin complex is a requisite step in elicitin recognition by receptors [26], we have still no indication on the importance of lipid–LTP complexes formation for binding to specific sites and/or for triggering cell responses. Moreover, the nature of extracellular or membrane putative ligands for LTP1 remains to be elucidated, since these proteins do not capture phytosterols as elicitins do.

In addition, these results address a major question about the structural motifs common to these protein families and involved in their recognition. The amino acid sequence of the wheat endosperm LTP1 displays 50% sequence identity with that of a putative LTP1 from the shoot apex of tobacco [27]. This is in the range (45–80%) obtained for the different LTPs 1 expressed in the leaves of *Arabidopsis thaliana* [28] and much higher than the identity (18%) found between cryptogein and wheat LTP1 (ALIGN software at Expasy). A common motif present on both LTP1 and elicitins could be postulated, but it could not be highlighted from sequence alignment. However, a structural alignment revealed interesting superimposition of some helices. Thus, to the helices H_A (8–20), H_D (56–66) and H_E (83–90) of cryptogein correspond the helix H_3 (44–56), H_1 (17–7) and H_2 (32–25) of wheat LTP1, respectively (Fig. 4). It is worthy to note that the superimposition is reversed (N-ter and C-ter directions) for H_D–H_1 and H_E–H_2. No superimposition but similar orientations are observed for helices H_C and H_4 of cryptogein and LTP1, respectively (Fig. 4). These similarities in the topology of helices displayed by cryptogein and wheat LTP1 could explain their similar affinity and competitiveness for the membrane receptor.

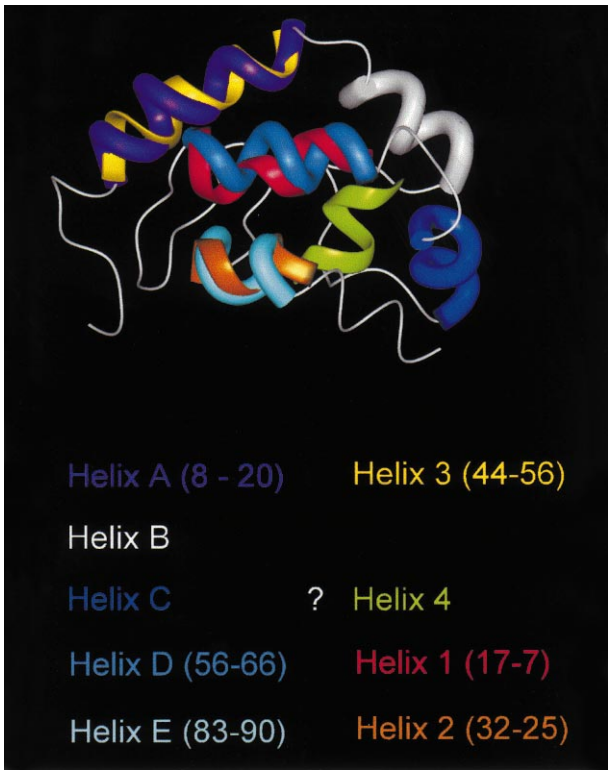

Fig. 4. Superimposition of cryptogein and wheat LTP1 backbones. The whole backbone of cryptogein (pdb entry: 1bxm) is presented while only the superimposed helices of LTP1 (pdb entry: 1gh1), or equivalent (for helix H_4), are displayed (top), using the INSIGHT II software (Accelerys, USA). Tubes and flat ribbons represent the helices of cryptogein and wheat LTP1, respectively (for helix E only the superimposed part is presented as a tube). The corresponding residues of superimposed helices or helix domain are detailed (bottom). To the D and E helices of cryptogein correspond the 1 and 2 helices of wheat LTP1, in the opposite orientation.

Finally, LTPs 1 are ubiquitous in the plant kingdom. In the same way, LTP or elicitin receptors were found in all plants assayed [8,25], although most of them do not develop a hypersensitive reaction after elicitin treatment [8]. It suggests that these receptors could be associated to a general mechanism involving LTP in a warning system able to detect exogenous organisms. Moreover, since elicitins trigger a hypersensitive reaction leading to the release of different mediators and molecules from cells, in a way comparable with that observed in severe allergy [29], it would be interesting to study if pan-allergen LTPs 1 of plant-derived foods could interact with animal specific receptors and if these receptors belong to the same family as that found in plants. Recognition by such receptors could be a first step in the cascade of metabolic pathways originating the allergenic response to plant LTPs 1. It should stimulate further investigations towards the evolutionary relationships between the hypersensitive reactions in both allergy and plant defence responses.

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References

[1] Garcia-Olmedo, F., Molina, A., Segura, A. and Moreno, M. (1995) Trends Microbiol. 3, 72–74.
[2] Kader, J.-C. (1996) Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 627–654.
[3] Broekaert, W.F., Cammue, B.P.A., De Bolle, M.F.C., Thevissen, K., De Samblanx, G.W. and Osborn, R.W. (1997) Crit. Rev. Plant Sci. 16, 297–323.
[4] Douliez, J.P., Michon, T., Elmorjani, K. and Marion, D. (2000) J. Cereal Sci. 32, 1–20.
[5] Pato, C., Le Borgne, M., Le Baut, G., Le Pape, P., Marion, D. and Douliez, J.P. (2001) Biochem. Pharmacol. 62, 555–560.
[6] Asero, R. et al. (2000) Int. Arch. Allergy Immunol. 122, 20–32.
[7] Hoffmann-Sommergruber, K. (2000) Int. Arch. Allergy Immunol. 122, 155–166.
[8] Ponchet, M. et al. (1999) Cell. Mol. Life Sci. 56, 1020–1047.
[9] Boissy, G., O'Donohue, M., Gaudemer, O., Perez, V., Pernollet, J.C. and Brunie, S. (1999) Protein Sci. 8, 1191–1199.
[10] Mikes, V., Milat, M.-L., Ponchet, M., Panabières, F., Ricci, P. and Blein, J.-P. (1998) Biochem. Biophys. Res. Commun. 245, 133–139.
[11] Hendrix, J.W. (1970) Annu. Rev. Phytopathol. 8, 111–130.
[12] Osman, H. et al. (2001) FEBS Lett. 489, 55–58.
[13] Keller, H., Blein, J.P., Bonnet, P. and Ricci, P. (1996) Plant Physiol. 110, 365–376.
[14] Kieffer, F., Lherminier, J., Simon-Plas, F., Nicole, M., Paynot, M., Elmayan, T. and Blein, J.-P. (2000) J. Exp. Bot. 51, 1799–1811.
[15] Hollenbach, B., Schreiber, L., Hartung, W. and Dietz, K.J. (1997) Planta 203, 9–19.
[16] Ricci, P. et al. (1989) Eur. J. Biochem. 183, 555–563.
[17] Charvolin, D., Douliez, J.P., Marion, D., Cohen-Addad, C. and Pebay-Peyroula, E. (1999) Eur. J. Biochem. 264, 562–568.
[18] Rustérucci, C., Stallaert, V., Milat, M.-L., Pugin, A., Ricci, P. and Blein, J.-P. (1996) Plant Physiol. 111, 885–891.
[19] Simon-Plas, F., Rustérucci, C., Milat, M.-L., Humbert, C., Montillet, J.-L. and Blein, J.-P. (1997) Plant Cell Environ. 20, 1573–1579.
[20] Blein, J.-P., Milat, M.-L. and Ricci, P. (1991) Plant Physiol. 95, 486–491.
[21] Vauthrin, S., Mikes, V., Milat, M.-L., Ponchet, M., Maume, B., Osman, H. and Blein, J.-P. (1999) Biochim. Biophys. Acta 1419, 335–342.
[22] Wendehenne, D., Binet, M.-N., Blein, J.-P., Ricci, P. and Pugin, A. (1995) FEBS Lett. 374, 203–207.
[23] Monod, J., Wyman, J. and Changeux, J.-P. (1965) J. Mol. Biol. 12, 88–118.
[24] Bourque, S., Ponchet, M., Binet, M.N., Ricci, P., Pugin, A. and Lebrun-Garcia, A. (1998) Plant Physiol. 118, 1317–1326.
[25] Bourque, S., Binet, M.-N., Ponchet, M., Pugin, A. and Lebrun-Garcia, A. (1999) J. Biol. Chem. 274, 34699–34705.
[26] Osman, H. et al. (2001) Mol. Biol. Cell 12, 2825–2834.
[27] Fleming, A.J., Mandel, T., Hofman, S., Sterk, P., De Vries, S.C. and Kuhlemeier, C. (1992) Plant J. 2, 855–862.
[28] Arondel, V., Vergnolle, C., Cantrel, C. and Kader, J.C. (2000) Plant Sci. 157, 1–12.
[29] Leung, D.Y.M. (1998) Mol. Genet. Metab. 63, 157–167.