Tunable Membrane Binding of the Intrinsically Disordered Dehydrin Lti30, a Cold-Induced Plant Stress Protein

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Dehydrins are intrinsically disordered plant proteins whose expression is upregulated under conditions of desiccation and cold stress. Their molecular function in ensuring plant survival is not yet known, but several studies suggest their involvement in membrane stabilization. The dehydrins are characterized by a broad repertoire of conserved and repetitive sequences, out of which the archetypical K-segment has been implicated in membrane binding. To elucidate the molecular mechanism of these K-segments, we examined the interaction between lipid membranes and a dehydrin with a basic functional sequence composition: Lti30, comprising only K-segments. Our results show that Lti30 interacts electrostatically with vesicles of both zwitterionic (phosphatidyl choline) and negatively charged phospholipids (phosphatidyl glycerol, phosphatidyl serine, and phosphatidic acid) with a stronger binding to membranes with high negative surface potential. The membrane interaction lowers the temperature of the main lipid phase transition, consistent with Lti30’s proposed role in cold tolerance. Moreover, the membrane binding promotes the assembly of lipid vesicles into large and easily distinguishable aggregates. Using these aggregates as binding markers, we identify three factors that regulate the lipid interaction of Lti30 in vitro: (1) a pH dependent His on/off switch, (2) phosphorylation by protein kinase C, and (3) reversal of membrane binding by proteolytic digest.

INTRODUCTION

Dehydrins constitute a group of intrinsically disordered plant proteins involved in the tolerance to cold and drought stress. The molecular mechanism behind their function is not yet established. From studies of other systems, it has become apparent that, despite the lack of a fixed three-dimensional structure, disordered proteins are often involved in key cellular processes such as signal transduction and stabilization of both proteins and RNA (Tompa, 2002; Dyson and Wright, 2005; Fink, 2005; Radiovojac et al., 2007; Dunker et al., 2008; Uversky and Dunker, 2010). Binding of a disordered protein typically induces folding and activation (Moham et al., 2006; Tompa and Fuxreiter, 2008; Wright and Dyson, 2009). However, there are also examples of binding without an appreciable degree of folding, or just local secondary-structure formation, for example, the binding of disordered T cell receptors to lipid vesicles (Sigalov and Hendrickx, 2009) and Cdc4 binding to Sic1 (Borg et al., 2007). The most obvious hint about the dehydrin molecular action is their characteristic content of repetitive and highly conserved sequence segments (Figure 1). Combined with their unusually high proportion of hydrophilic and charged amino acids, this modular sequence pattern makes them unsuitable for adapting a specific hydrophobic core (Figure 1). Dehydrins are found to be highly resistant to unspecific chain collapse in vitro (Mouillon et al., 2008). Taken together, this suggests a functional adaptation to remain coil like in the highly crowded cytosol of desiccated plant cells, most likely to assure maximum exposure of the local, conserved segments to their biological targets (Mouillon et al., 2006, 2008). In accordance with these sequence characteristics, an early hypothesis has been that dehydrins interact as a group with cellular membranes and modulate their properties via the characteristic K-segments (Dure, 1993; Close, 1996). However, experimental tests of this idea have generated conflicting results. Favoring membrane binding, some dehydrins are found to colocalize with membrane surfaces in stressed plant cells (Danyluk et al., 1998; Puhakainen et al., 2004). Moreover, the maize (Zea mays) dehydrin DHN1 (YSK2; see Figure 1 for nomenclature) and the two Arabidopsis thaliana dehydrins, Lti29 and Erd14, are also found to interact with liposomes in vitro (Koag et al., 2003; Kovacs et al., 2008; Koag et al., 2009). Even so, yet other dehydrins (e.g., the soybean [Glycine max] DHN1 [Y2K]; Soulages et al., 2003) are not observed to bind lipid vesicles under corresponding conditions. As the K-segment is a common feature of all these proteins, data show that this segment alone is not a useful indicator of membrane binding. The question then arises: Is the proposed role of the K-segment in membrane binding unjustified or could there be additional...
regulating sequence factors at play? In this study, we identify precisely such a sequence factor: flanks of His side chains that regulate the interactions between the K-segments and membranes in a pH-dependent manner.

RESULTS

Surface Plasmon Resonance: Lti30 Displays High Affinity to Membrane Vesicles

As a sensible probe for Lti30 binding to lipid membranes, we used surface plasmon resonance (Biacore). Following standard protocols, lipid vesicles were immobilized on a lipid binding Biacore chip, and Lti30 (10 μM) was allowed to flow over the surface. The chip was divided into four detection areas, allowing the simultaneous study of three vesicle-coated surfaces and a control. The results are presented in Figure 2 as Biacore sensograms, showing the transfer of dehydrin mass to the surface in response units (RUs). Binding of Lti30 to vesicles of dioleic phosphatidyl glycerol (DOPG; negatively charged), dioleic phosphatidyl serine (DOPS; negatively charged), and dioleic phosphatidyl choline (DOPC; neutral zwitterionic) was confirmed by a pronounced increase in RU (>600). The observed affinity is high since a subsequent wash step with buffer was not sufficient to completely reverse the Lti30 binding (Figure 2). The molecules that dissociate during this wash step represent most likely a subfraction of loosely bound material (i.e., the interaction with the Biacore surface is not perfectly uniform). It is nevertheless clear that Lti30 binds most extensively to the negatively charged vesicles composed of DOPG and DOPS (Figure 2). This binding becomes progressively weaker as the net negative charge of the vesicles is decreased by increasing the fraction of neutral DOPC in the mixed lipid vesicles. Going from a 3:1 mixture of DOPC: DOPG to pure DOPC vesicles decreases the Lti30 binding by 25%. The sensitivity to vesicle charge indicates that electrostatic forces modulate Lti30 binding: the positively charged amino acids of the disordered protein are attracted to the negatively charged membrane surface. To test this interpretation, we altered the positive charge of Lti30 by changing the buffer pH. Consistently, the introduction of more positive charges at pH 4.0 augments the protein-vesicle interactions, and, vice versa, the depletion of positive side chain charges at pH 9.0 suppresses the binding drastically (Figure 2C). Notably, short peptides of the canonical K-segment (EKKGIMDKIKLEKPG) did not bind to any of the lipids (see Supplemental Figure 1 online), implying that residues outside the K-segments are critical for determining the membrane affinity.

Solid-State 31P MAS NMR: Interaction of Lti30 with Vesicles Requires Positively Charged Residues

To obtain more detailed molecular information about the interaction between Lti30 and negatively charged vesicles, we employed high-resolution solid-state 31P magic angel spinning NMR spectroscopy (Lindström et al., 2005). This technique provides, at the molecular level, information for each lipid component separately and allows the detection of even very small changes in the local electrostatic environment of their headgroup region (Kooijman et al., 2007). Lti30 was added to dimyristoyl-phosphatidylcholine:dimyristoyl-phosphatidylserine (DMPC:DMPS; 3:1 molar ratio) vesicles at a ratio of 1:100, and
the ionic strength was kept at a minimum. The change in fatty acids from dioleoyl (DO) to dimyristoyl (DM) in these NMR experiments was done to enable a direct comparison with our complementary calorimetric studies of the phase behavior of these membranes, which requires lipids with phase transition temperatures above 273K in an aqueous environment. Notably, this change in fatty acid composition has no significant impact on the interaction with Lti30 as controlled by Biacore. Figure 3 displays the NMR spectra obtained for these lipid systems prior and after addition of Lti30. The presence of the peptide induces a pronounced perturbation for both lipid resonances. The observation indicates a pure electrostatic charge compensation mechanism upon binding of the Lti30 peptide via its positively charged residues to the negatively charged vesicles (Lindström et al., 2005). DMPS shows here the largest shift since it carries the net negative charge. The DMPS peak shifts upfield by 0.3 ppm, and for DMPC a weaker upfield shift of 0.1 ppm is observed. The observation of an electrostatically driven Lti30–membrane interaction agrees well with studies on other vesicle binding disordered proteins, such as the T cell receptor (Sigalov and Hendricks, 2009), the viral genome–linked protein (Vpg) (Rantalainen et al., 2009), and α-synuclein (Davidson et al., 1998; Beyer, 2007). While Lti30 exhibits a weak affinity for neutral vesicles made of zwitterionic DOPC, presumably due to weak hydrophobic interactions, Lti30 also has a very pronounced interaction with lipids containing the negatively phosphatidic acid DOPA (data not shown). Solid-state analysis of how proteins bind to membranes containing negatively charged lipids, such as phosphatidylserine, phosphatidylglycerol (PG), and phosphatic acid (PA), has been undertaken by several groups (Pinheiro and Watts, 1994; Lindström et al., 2005; Jack et al., 2008). Consistent with our data, they all see that proteins bind quite unspecifically to the negatively charged membrane surface, without forming specific interactions with the individual lipids. However, Kooijman et al. (2007) identified specific protein–lipid interactions in the presence of PA, where positive side chains bond electrostatically to the lipid phosphate group, inducing a formal negative charge of −2. On this basis, we deduce that the positive amino acids of Lti30, which can only be H or K, coordinate in a similar way with the PA used in our Biacore experiments. Interestingly, the distribution of the positively charged residues in the Lti30 sequence coincides almost precisely with the position of the K-segments. Outside the His-flanked K-segments, there are only two positive charges: one at the isolated Lys-159 and one at the N terminus (Figure 1).
Differential Scanning Calorimetry: Lti30 Binding Modulates the Temperature Interval of the Membrane’s Functional Phase

Here, we used differential scanning calorimetry (DSC) experiments (Ivanova et al., 2003) to study the impact of Lti30 on the lipid phase behavior of mixed DMPC:DMPG vesicles at a 3:1 molar ratio. In Figure 4, the split peak reflects the phase transition of the two individual lipids, from a gel phase toward the biologically viable liquid crystalline phase at elevated temperature. Addition of Lti30 to the vesicles induces a distinct change in the DSC thermograms of the lipid bilayer: the previously split phase transition becomes homogeneous and shifts down by 2.5°C (Figure 4). The biologically functional liquid crystalline phase is able to persist at lower temperatures. Similar shifts of the phase transition temperature (Tm) have been seen in the presence of other proteins or peptides (Cseh et al., 2000; Ivanova et al., 2003; Pedersen et al., 2005). Upon increasing the Lti30 to lipid fraction to a 1:30 molar ratio, the transition temperature drops even further (see Supplemental Figure 2 online). Dehydrins that do not bind to vesicles (e.g., Cor47) have no effect on the DSC thermograms (see Supplemental Figure 2 online). The detection of vesicle binding by DSC is thus in good agreement with data obtained by Biacore and solid-state NMR (Figures 2 and 3). Moreover, the decreased phase transition temperature induced by Lti30 is, at least qualitatively, consistent with the proposed physiological role of the protein (i.e., the protein extends the functional lamellar phase of the membrane toward lower temperatures).

Lti30 Assembles Lipid Vesicles and Thylakoid Membranes into Aggregates: A Possible Role in Membrane Cross-Linking

As final evidence for membrane binding, we find that Lti30 assembles large unilamellar vesicles (LUVs; 100 nm) of palmitoyl oleoyl phosphatidyl choline (POPC):palmitoyl oleoyl phosphatidyl glycerol (POPG) (1:3 molar ratio) into macroscopic aggregates (Figure 5). The emergence of the Lti30 vesicle aggregates can be followed directly by light microscopy, light scattering, and sometimes even by the naked eye. Interestingly, this ability of Lti30 to promote membrane assembly is not limited to model membranes but is also observed with biological material. Repeating our experiments with thylakoid membranes isolated from spinach (Spinacia oleracea) yields indistinguishable results (Figure 5). In contrast with the LUVs, thylakoid membranes are composed mainly of uncharged galactolipids and contain <10% phospholipids (Mackender and Leech, 1974). This suggests that the interaction between Lti30 and the thylakoid membranes relies on (or induces) local regions of negatively charged phospholipids. It is also conceivable that Lti30 has an affinity for some of the negatively charged proteins that are dispersed in high numbers in the thylakoid membranes (Barber, 1982). The ability of Lti30 to assemble synthetic vesicles and ex vivo membranes into large aggregates has not been reported before, but other LEA proteins have recently been shown to have similar ability (Božović, 2007; Hundertmark et al., 2011). However, the mitochondrial LEA protein LEAM has been observed to bind and stabilize lipid vesicles upon drying, but aggregation is not reported to accompany the process (Tolleter et al., 2007). Likewise, the membrane binding dehydrin DHN1 from maize seems to lack the ability to aggregate vesicles since circular dichroism (CD) spectra in the presence of vesicles could be monitored without disturbances, indicating well dispersed solutions (Soulages et al., 2003; Koag et al., 2009). Besides pointing to a possible physiological role of Lti30, the aggregation phenomenon in Figure 5 provides a sensitive and handy marker for examining how the protein’s membrane affinity is modulated by external factors.

Figure 4. DSC of Lti30, Phosphorylated Lti30, and DMPC:DMPG (3:1 Molar Ratio) Vesicles.

DMPC:DMPG vesicles alone (middle) and in the presence of Lti30 (left) or of phosphorylated Lti30 (right) (lipid-to-protein 100:1 molar ratio in both cases). Lti30 reduces the phase transition temperature of the lipid vesicles by ~2.5°C. A direct opposite response to this is found by binding of the phosphorylated Lti30 that causes an increase in the phase temperature.

Figure 5. Pictures (Light Microscopy) of Lti30 together with LUVs or Thylakoids Showing the Formation of Aggregates.

(A) POPC:POPG (3:1 molar ratio) LUVs (1.4 mM) alone at pH 6.3.
(B) Lti30 (14 μM) and POPC:POPG LUVs (1.4 mM) at pH 6.3.
(C) Spinach thylakoid membranes alone (0.2 mg/mL).
(D) Lti30 (0.2 mg/mL) and spinach thylakoids (0.2 mg/mL) assembled into large aggregates.
The K-Segment Is Flanked by Protonable His Residues: A Putative Modulator of Membrane Binding

Since not all proteins in the dehydrin family associate with membranes, it is reasonable to assume that the presence of K-segments alone is not sufficient for lipid binding. Consistently, the synthetic peptide EKKGIMDKIKEKLPG, representing the canonical sequence of an isolated K-segment, shows no indication of coordinating vesicles in Biacore experiments (see Supplemental Figure 1 online) or in light microscopy assays (see Supplemental Figure 3 online). Upon closer analysis of the Lti30 sequence, however, it can be seen that the K-segments are, without exception, flanked by pairs of His residues. In five cases, these His pairs are found at both sides of the K-segment and in one case at the C-terminal end only (Figure 1). His residues are also seen to colocalize in varying patterns with K-segments in other proteins of the dehydrin family (see Supplemental Tables 1 and 2 online). The question is then whether these His residues have any role in augmenting membrane affinity. Of particular interest here is that His side chains have intrinsic $pK_a$ values of around 6.5 and thus readily undergo protonation and deprotonation reactions at physiological pH values. Such protonation could facilitate the binding to negative lipids in two ways. Locally, by making the local electrostatic environment around the individual K-segments more positive at low pH and globally by increasing the net positive charge of Lti30. To test this idea, Biacore and vesicle aggregation experiments were performed at three different pH values. Our previous data at pH 6.3, where the His residues are expected to be partly protonated, were complemented with experiments at pH 4.0 and 9.0, where the His residues are fully protonated or deprotonated, respectively. The Biacore analysis shows that decreased pH augments the binding of Lti30 to the negatively charged membranes, whereas increased pH abolishes it completely (Figure 2C). The same trend can be observed in the vesicle aggregation assays with Lti30, where large aggregates form readily at pH 4.3 but vanish at pH 9.0 (Figure 6). Subsequent centrifugation of the aggregated material shows that Lti30 copellets with the aggregates at pH 4.3, 6.3, and 7.2 but not at pH 9.0 (Figure 6). The ability of Lti30 to aggregate vesicles seems thus to correlate with protonation of His residues.

Minimal Formalism for Lti30 Membrane Binding

To analyze quantitatively the pH dependence of Lti30 binding to lipid membranes ($\text{lip}^-$), we assumed a binding model where protonated Lti30 has a higher affinity to $\text{lip}^-$ ($K_{d}^\text{H} = [\text{Lti30}^\text{H}]/[\text{Lti30}^\text{H} \cdot \text{lip}^-]/[\text{Lti30}^\text{H} \cdot \text{lip}^-]$) than nonprotonated Lti30 ($K_{d} = [\text{Lti30}]/[\text{Lti30} \cdot \text{lip}^-]/[\text{Lti30} \cdot \text{lip}^-]$) (i.e., $K_{d}^\text{H} < K_{d}$) (Figure 7). It then follows from mass action (Oliveberg et al., 1994, 1995) that

$$K_{d}^{\text{bound}} = K_{d}^{\text{free}} = pK_a^{\text{bound}} - pK_a^{\text{free}} = pK_a^{\text{H}} - pK_a$$ (1)

where $pK_a^{\text{bound}}$ and $pK_a^{\text{free}}$ are the $pK_a$ values for membrane-bound and free Lti30, respectively. Equation 1 shows that an increase in membrane affinity is always coupled to an increase of the $pK_a$ value of the bound protein species (i.e., $pK_a^{\text{bound}} > pK_a^{\text{free}}$). In other words, the interaction with the negatively charged lipid leads to a stabilization of the protonated, positively charged form of Lti30. It also follows that the observed affinity between Lti30 and $\text{lip}^-$ ($K_{d}^{\text{obs}} = [\text{Lti30}^\text{H} \cdot \text{lip}^-]/[\text{Lti30}^\text{H} \cdot \text{lip}^- + \text{Lti30} \cdot \text{lip}^-]$) describes a pH dependence (Oliveberg et al., 1994), as seen in Equation 2,

$$\frac{d\log K_{d}^{\text{obs}}(pH)}{dpH} = Q_{\text{bound}}(pH) - Q_{\text{free}}(pH) = \Delta Q(pH)$$ (2)

where $\Delta Q(pH)$ is the number of $\text{H}^+$ exchanged upon membrane binding at each given pH value. Accordingly, the membrane

Figure 6. Effect of pH on Lti30-Induced Vesicle Aggregation.

(A) to (D) Lti30 (14 μM) and DOPC:DOPG (total of 1.4 mM at 3:1 molar ratio) LUVs at pH 4.3 (A), pH 6.3 (B), pH 7.2 (C), or pH 9.0 (D).

(E) SDS gel showing the amount of Lti30 in the vesicle pellet (v) as a function of pH. For comparison, the second lane (s) indicates the level of protein in the supernatant. Notably, the intensities of the v and s lanes do not sum up to the total protein content of 0.2 mg Lti30, as only 25% of the supernatant volume was loaded to the gel; the material loaded to the v lanes, by contrast, contains 100% of the vesicle-bound protein.

(F) Amount of total protein in pellet and supernatant at the different pH values. Lti30 (0.2 mg; 9.3 μM) was added to 0.93 mM DOPC:DOPG LUVs (3:1 molar ratio) in a total volume of 100 μL.
affinity is predicted to show a constant value of $K_d$ at pH values below $pK_{A_{\text{free}}}$, where both the free and bound forms of $Lti30$ are protonated and, conversely, a constant value of $K_d$ at pH values of above $pK_{A_{\text{bound}}}$, where both the free and bound forms of $Lti30$ are nonprotonated. At pH values between these stationary regimes, the affinity changes from $K_d^{\text{H}+}$ to $K_d^{\text{H}-}$, with characteristic kinks around $pK_{A_{\text{free}}}$ and $pK_{A_{\text{bound}}}$ (Figure 7).

The pH Dependence of Vesicle Aggregation Corroborates the Involvement of the Flanking His Residues: A His Switch for Regulation of Membrane Adhesion

As an experimental measure of how the membrane affinity of $Lti30$ changes with pH, we used the concentration of $Lti30$ at which a predefined degree of vesicle aggregation is obtained. PC:PG vesicles (1.4 mM lipid at 3:1 molar ratio) equilibrated at different pH were titrated with $Lti30$, and the extent of vesicle aggregation was measured by absorbance at 400 nm, which is inversely proportional to the extent of light scattering (Figure 7). The $Lti30$ concentration at which the absorbance exceeded 0.5 was denoted $[Lti30]^{0.5}$ and plotted versus pH (Figure 7). All titrations followed the same time protocol to cancel kinetic effects and to produce a function of $Lti30^{0.5}$ versus pH that is as far as possible proportional to $K_d^{\text{H}+}(pH)$ in Equation 2. The resulting plot of $Lti30^{0.5}$ shows good agreement with the binding model in Figure 7B and yields a $pK_{A_{\text{free}}}$ value of around 6.5. This value matches precisely that of a free His side chain. It can also be noted that the corresponding effect of the acidic residues Asp and Glu, which protonate around pH 4.5, seems too small to be resolved. Moreover, since the plot does not level out below pH 9.0 (Figure 7D), we conclude that $pK_{A_{\text{bound}}}$ > 8 and, correspondingly, that $pK_{A_{\text{bound}}}-pK_{A_{\text{free}}}=pK_d^{\text{H}+}-pK_d > 1.5$ (Equation 1). Similar $pK_{A}$ shifts are found for salt bridges in proteins (Oliveberg et al., 1995; Vaughan et al., 2002) and for the His of the FYVE domain upon binding to the negatively charged lipid phosphatidylinositol (Lee et al., 2005). Determination of precisely how many H+ are exchanged in the $Lti30$ binding process is yet precluded by our approximate estimate $K_d^{\text{H}+}(pH)$: Even so, these data provide direct evidence that the interaction between $Lti30$ and membranes is indeed modulated by the ionization states of the flanking His residues. Notably, there is no effect of Asp and Glu protonation around their expected $pK_{A}$ values at pH 4.3. The explanation could be that these residues cannot salt-link to the negative membrane charges in their protonated form where they become neutral. Also, there is no indication of protonation of the actual lipids in the titration data, consistent with the apparent $pK_{A}$ values of PG and PC vesicles of < 3 (Watts et al., 1978; Hanahan, 1997). For an unambiguous identification of the sequence segments of $Lti30$ that serve to assemble the vesicles, we added flanking His residues to the canonical K-segments in the form of the synthetic peptide HHEKKGM-TEKVMEKIQEQLPGHH. Addition of this isolated His-flanked K-segment to PC:PG vesicles at pH 4.3 induces aggregation indistinguishable from that of the full-length protein (see

Figure 7. The pH Dependence of $Lti30$ Membrane Binding Shows the Involvement of His Protonation.

(A) Coupled equilibria describing the pH dependence of the $Lti30$ lipid binding (cf. Equations 1 and 2).

(B) The pH dependence of the $Lti30$ lipid affinity, calculated from the equilibria in (A) (Equations 1 and 2). The affinity changes between the $pK_{A}$ values of $Lti30$ in its free ($pK_{A_{\text{free}}}$) and membrane-bound state ($pK_{A_{\text{bound}}}$).

(C) The binding of $Lti30$ to lipids measured by lipid aggregation (absorbance at 400 nm) versus protein concentration, at pH values between 4.0 and 9.0. The changes in affinity versus pH was derived from the $Lti30$ concentration where the absorbance equals 0.5 ($Lti30^{0.5}$; dotted line).

(D) The observed pH dependence of the affinity between $Lti30$ and lipids derived from experimental data in (C). Following the formalism in (A) and (B), the $pK_{A}$ value of unbound $Lti30$ is estimated to around 6.5, in good agreement with the $pK_{A}$ value of free His. The $pK_{A}$ value of lipid-associated $Lti30$ is not clearly resolved in the titration range, and hence >8 to 9.
Supplemental Figure 4 online). On this basis, we conclude that the sequence motif governing the interaction between Lti30 and membranes consists of two components: a K-segment in combination with a pH-dependent switch of flanking His residues.

**Phosphorylation Assay: Membrane Binding of Lti30 Is Modulated by Phosphorylation**

Phosphorylation of the dehydrins is observed to take place both in vivo and in vitro, indicating a role in functional regulation in stressed plant cells (Alsheikh et al., 2003; Jiang and Wang, 2004; Röhrig et al., 2006; Brini et al., 2007). The sequence algorithm Netphos (Exasy) predicts that Lti30 is specifically phosphorylated by protein kinase C (PKC) at nine different positions, several of which are in the K-segments (Figure 1). It is interesting, however, that the Lti30 sequence shows no hits for the alternative casein kinase II (CKII), which has previously been found to phosphorylate another class of dehydrins, namely, those with S-segments (Table 1). Consistent with the predictions, we observe that Lti30 easily becomes phosphorylated by PKC as detected by radiolabeled phosphate (Figure 8). With CKII, we observe no corresponding effect, consistent with previous reports from other groups (Alsheikh et al., 2005). We previously showed that phosphorylation has no detectable effect on the structures of solubilized dehydrins (Mouillon et al., 2008), and, in line with this, CD analysis of Lti30 reveals no structural changes upon phosphorylation (see Supplemental Figure 5 online). Nevertheless, we see here that phosphorylation of Lti30 significantly affects the protein’s ability to assemble vesicles in vitro (Figure 8). Clearly, it prevents the formation of large vesicle aggregates as detected by light microscopy (Figure 8). Consistently, phosphorylation has earlier been reported to produce electrostatic off-switches that could serve to counteract those of His protonation in Figure 7. For example, phosphorylation of Ser residues within a cluster of positively charged amino acids reverses membrane binding of the disordered MARCKS protein (McLaughlin and Aderem, 1995). In contrast with the effect of increased pH, phosphorylation of Lti30 does not completely inhibit vesicle aggregate formation but seems rather to limit the size of the aggregates (Figure 8). The aggregation behavior indicates that phosphorylation of Lti30 even facilitates the formation of small vesicular clusters, perhaps by preventing them from assembling into larger

Table 1. Physical-Chemical Properties and Sequence Characteristics of Dehydrins from Different Subgroups

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Isoelectric point (pl), number of positive amino acids (+amino acid), number of negative amino acids (−amino acid), total number of His residues in the sequence (His), net charge at pH 6.0 assuming that 50% of the His residues are protonated (charge pH 6), net charge at pH 7.0 assuming that 20% of the His residues are protonated (charge pH 7), and number of PKC, CKII, and other (other) phosphorylations sites predicted by NetPhosK (probability limit set to 60%, Exasy tools). It can be seen that K<sub>n</sub>-dehydrin is almost exclusively phosphorylated by PKC. The number of PKC sites in the different classes of dehydrins scales K<sub>n</sub>> Y<sub>2SKK</sub> > SK<sub>n</sub> and the number of CKII sites SK<sub>K</sub> >> Y<sub>2SKK</sub> >> K<sub>n</sub>.
and DOPG (3:1 Molar Ratio) LUVs at pH 6.3 (Protein-to-Lipid Ratio 1:100).

Membrane-bound Lti30 was monitored both by SDS gels and by digestion of Lti30 was detected by SDS gel electrophoresis, and digestion of soluble trypsin, degradation of free Lti30 was tested in parallel with digestion of soluble Lti30 bound to membranes. Digestion of soluble Lti30 is readily degraded by trypsin in both its free and membrane-bound states (Figure 9). This corroborates the idea that Lti30 interacts mainly with the surface of the membrane and does not become buried upon association. Moreover, trypsin is a lys-specific protease, and in Lti30, the Lys residues are found exclusively within the K-segments. This means that only the K-segments are cleaved by trypsin and, as a consequence, the vesicle aggregates dissolve. Besides pointing at proteolytic cleavage as a functional regulator of Lti30, these data provide additional evidence that the actual interaction between Lti30 and membranes involves the K-segment.

**DISCUSSION**

**Membrane Binding of Lti30 Is Regulated by a pH-Dependent His Switch**

The binding of dehydrins to membranes was proposed to be facilitated by their characteristic and highly conserved K-segments (Close, 1996; Koag et al., 2009). Consistently, the Lti30 (K2) dehydrin analyzed in this study was found to localize preferentially at membrane surfaces in electron micrographs (Danyluk et al., 1998; Puhakainen et al., 2004). Moreover, dehydrins such as Lti29 (SK2), Erd14 (SK2) (Kovacs et al., 2008), and DHN1 from maize (YSK2) (Koag et al., 2003, 2009) were found to coelute with lipid vesicles in vitro. When the K-segments of DHN1 were removed by mutation, the truncated versions of the protein displayed reduced ability to associate with the lipid vesicles (Koag et al., 2009). By contrast, the dehydrin rGMDH1 from soybean (YSK2) did not bind to any kind of lipid vesicles despite containing the characteristic K-segments (Soulagés et al., 2003), an observation that challenged the generality of the membrane binding capacity. The results on Lti30 presented in this study seem to reconcile these conflicting observations: the K-segments are not alone responsible for the membrane binding but rely also on the ionization state of their flanking His residues (Figures 2, 6, and 7; see Supplemental Figure 4 online). These flanking His residues act as a pH-dependent switch that modulates the protein’s membrane affinity (Figure 10) (i.e., the His residues switch from being neutral to positively charged, thereby increasing the electrostatic attraction to the membrane surface) (Table 1). Colocalization of His residues and K-segments is not unique to Lti30 but is also found to varying degrees in other dehydrins (see Supplemental Tables 1 and 2 online). Possibly, the varying pattern of His flanking is coupled to varying membrane affinity of the individual K-segment. As the pH values of the His shifts from 6.5 to >8.0 upon membrane binding, the switch becomes most responsive in the physiological pH range (Figure 7). An analogous mechanism has been reported for the globular phosphoinositide binding domain 1 (EEA1), where membrane targeting is engaged by an acidic cellular environment (Lee et al., 2005). Depending on the protonation states of two neighboring His residues at the EEA1 surface, the protein changes from cytosolic to membrane bound (Lee et al., 2005). The principal difference is that, in Lti30, the ordered lipid binding surface of EEA1 is substituted by modular K-segments flanked...
by His residues. Given the electrostatic nature of the dehydrin-membrane interaction, it is expected that also the global charge of the protein will influence the affinity. Although functional colocalization of His switches and K-segments, as displayed by Lti30, is likely to be favored evolutionarily by gene duplication and fragment insertion, it is conceivable that the different members of the dehydrin family also needs to be tuned globally to different windows of membrane affinity depending on their individual roles in the stress response. The existence of such global tuning is apparent upon comparison of the two dehydrins DHN1 and rGMDHN1 (Table 1; see Supplemental Figure 7 and Supplemental Tables 1 and 2 online). Even though both of these proteins comprise His-flanked K-segments, the overall positively charged DHN1 binds negatively charged vesicles with high affinity (Koag et al., 2009), whereas the negatively charged rGMDHN1 remains unbound and monomeric (Soulages et al., 2003) (Table 1). Global charge can thus act as a decisive secondary regulator for membrane association (see Supplemental Figure 7 online); if the net negative repulsion between the membrane and the protein is too large, the local K-segments are prevented from binding, even if they are flanked by positively charged His residues (Table 1; see Supplemental Tables 1 and 2 online). Equipped with this simple rule of thumb, a distinct pattern emerges upon comparison of the different dehydrin classes (Table 1). The dehydrins containing only K-segments (Kn), or combinations of Y-, S-, and K-segments (YnSKn), show an overall positive global charge, whereas the dehydrins containing just S- and K-segments (i.e., SKn) are all negative (Table 1). On this basis, it is reasonable to assume that most of the K_n and YSK_n dehydrins, but not the SK_n dehydrins, will associate with negatively charged membranes. If this turns out to be correct, such distinct membrane association properties could indicate a functional division within the dehydrin family.

**Tuning of the Membrane Properties by Lti30 Phosphorylation**

The second factor that modulates the association of Lti30 to membranes is phosphorylation. Out of nine phosphorylation sites in Lti30, three are located directly within K-segments and

![Figure 9. Trypsin Digestion of Lti30 Dissolves the POPC:POPG (3:1 Molar Ratio) LUV Aggregates.](image-url)
membranes. The Tm of the membrane phase transition increases at the same time alters the phase behavior of the vesicle (Figure 8). Interestingly, DSC data show that phosphorylated gates, which end up smaller than with nonphosphorylated Lti30 of phosphorylated Lti30 is also seen to alter the vesicle aggregate some of its membrane binding capacity. Membrane association intact K-segments explain why phosphorylated Lti30 still retains phosphorylated, the net global charge would decrease from +13 to +4 at neutral pH. This residual positive charge and the two intact K-segments explain why phosphorylated Lti30 still retains some of its membrane binding capacity. Membrane association of phosphorylated Lti30 is also seen to alter the vesicle aggregates, which end up smaller than with nonphosphorylated Lti30 (Figure 8). Interestingly, DSC data show that phosphorylated Lti30 at the same time alters the phase behavior of the vesicle membranes. The Tm of the membrane phase transition increases to 27°C, which is above the value of free vesicles (Figure 4). Thus, in contrast with nonmodified Lti30, phosphorylated Lti30 appears to decrease the fluidity of the lipid bilayer. Notably, this effect is similar to that observed upon membrane association of the disordered heat shock protein HSP12, a LEA-like protein from *Saccharomyces cerevisiae* (Welker et al., 2010): phosphorylation seems to change Lti30 from a cold shock protein that increases lipid fluidity to a heat shock protein that decreases lipid fluidity. Even if this resemblance may be accidental, the very phenomenon opens the possibility that the role of phosphorylation is to deactivate selectively and gradually the membrane fluidity effect of Lti30 binding. Along similar lines, stepwise phosphorylation of the disordered transcription factor Ets-1 is coupled to a graded DNA binding affinity, which functions as a rheostat in cell signaling (Pufall et al., 2005). Comparison of the different phosphorylation sites among the divergent proteins in Table 1 points again at a functional division within the dehydrin family. As demonstrated earlier, the *Arabidopsis* dehydrins Cor47 and Lti29 (both SKn dehydrins), but not Lti30, become phosphorylated in vitro by CKII (Riera et al., 2004; Alsheikh et al., 2005; Mouillon et al., 2008). The main reason for this different kinase selectivity is the lack of conserved multi-S-segments, which constitute the prime target for CKII activity, in the Kα dehydrins, such as Lti30. According to NetphosK predictor (Expasy), the amino acid sequences of the Kα dehydrins show a nearly complete lack of CKII sites. Instead, Lti30 and the other Kα dehydrins comprise several Thr and Ser sites with high propensity for phosphorylation by PKC. This bias in amino acid composition between the Kα, YαSKα, and SKα-dehydrins gives rise to a distinct difference in their kinase specificities: the Kα- and YαSKα-dehydrins are mainly targeted by PKC, whereas the SKα-dehydrins are mainly targeted by CKII (Table 1). Judging solely by the high negative charge of the SKα-dehydrins, it is difficult to conceive that addition of further negative charges through CKII phosphorylation will promote binding to negatively charged membrane surfaces. The biological targets for these dehydrins thus appear different. Although plants have substantially more kinases than considered here, one general conclusion can be drawn from the predictions in Table 1: the distinct kinase profiles of the different classes of dehydrins show that their biological function can be regulated separately. Further exploration of such selective regulation mechanisms needs to await data showing at the sequence level the phosphorylation sites employed under stress in vivo. Consistent with the model in Figure 10, however, protein phosphorylation has been observed to produce electrostatic off-switches similar to, but opposing, those of His residues. For example, phosphorylation of Ser residues within a cluster of positively charged amino acids reverses membrane binding of the disordered MARCKS protein (McLauglin and Aderem, 1995).

**LUVs Do Not Protect Lti30 against Protease Degradation, but Degradation Reverses LUV Aggregation in Vitro**

As expected for a disordered protein, the degradation of free Lti30 by trypsin is fast (Figure 9). Disordered proteins are on the whole found to be degraded ~100 times faster than folded proteins (Dunker et al., 2008; Kovacs et al., 2008; Rantalainen et al., 2009). Interestingly, our data show that binding of Lti30 to LUVs does not protect against degradation (Figure 9). This behavior contrasts with that of the disordered proteins Vpg and HSP12, which both become protected against degradation upon vesicle association (Rantalainen et al., 2009; Welker et al., 2010). Moreover, we find that proteolytic cleavage of membrane-associated Lti30 leads to dissociation of the vesicle aggregates: the large aggregates disappear gradually as Lti30 is degraded (Figure 9). This sensitivity to proteolytic cleavage suggests that Lti30 associates mainly with the membrane surface with high accessibility to the solvent molecules, in good accordance with the NMR data (Figure 3) and the maintained ability of the vesicles to contain calcein (see Supplemental Figure 3 online). Moreover, it is indicated by the selective localization of cleavage sites in the K-segments that the protease cleaves Lti30 at its membrane

![Figure 10. Model of Tunable Vesicle Binding by Lti30.](image-url)

(1) Protonation of His residues flanking the K-segment promotes binding and deprotonation reverses binding. (2) Phosphorylation of the K-segment modulates the interaction, changes the lipid phase transition, and leads to smaller and more dispersed vesicle aggregates.
anchoring points and spares the sequence regions that connect them. In terms of regulation, this cleavage pattern seems to constitute an efficient means of reversing the membrane association of Lti30. Since His-flanked peptides are able to aggregate vesicles on their own, targeting the connecting regions of the Lti30 sequence is unlikely to have any clearing effect. This selectivity of the proteolytic action puts it forward as an interesting candidate for reversal of the Lti30 membrane association in vivo. Although the model protease trypsin has not been reported in plants, the Arabidopsis genome encodes over 800 proteases, which are distributed over almost 60 families (van der Hoorn, 2008). For the majority of the plant proteases, the proteolytic activity and substrate specificity are yet unknown (van der Hoorn, 2008), but presumably several of these will have trypsin-like specificity. Some of the plant proteases are also expressed in direct response to water stress (Contour-Ansel et al., 2010). Even so, the role of proteolytic cleavage in modulating membrane binding of dehydrins in vivo remains at this stage speculative and needs further experimental evaluation. Taken together, this leaves us with three putative regulatory mechanisms for the Lti30 function under physiological conditions: (1) onset of membrane binding by protonation of His residues, (2) tuning of binding properties by phosphorylation, and (3) reversal of binding by protease/peptidase cleavage of the K-segments. The role of this interaction in stress tolerance could be to stabilize native membrane topology and integrity structurally by cross-linking and/or colloidal by modulating lipid fluidity. This rather simplistic model for Lti30 function raises some questions. If the linker regions of the Lti30 sequence are not needed for membrane association, why are the K-segments not expressed individually as shorter peptides? One possibility is that the connecting regions of the full-length protein have a geometrical role in spacing and assembly of the targeted membrane surfaces. Also, and mechanistically more clear-cut, the covalent linkage of multiple K-segments will enable a higher local concentration of active material by avoiding the high chemical potential associated with multiple separate K-segments. The latter factor could be particularly important under drought stress where the content of free water is decreased.

**METHODS**

**Expression and Harvesting**

Expression and purification of the recombinant *Arabidopsis thaliana* dehydrin Lti30 were performed according to Svensson et al. (2000), with the following minor changes. One-hundred-and-fifty microliters of glycerol stocks of the *Escherichia coli* strain were spread on Luria agar plates (150 μg ampicillin) and grown at 37°C overnight. Resuspended cells were then added to 2 liters of Luria-Bertani medium containing 50 μg/mL ampicillin. Expression was induced at an OD₆₀₀ of 0.6 by 1 mM isopropyl β-D-thiogalactopyranoside, and the cells were cultured at 37°C for 4 h. Cells were harvested by centrifugation at 6000 rpm for 15 min and the pellet stored at −20°C. The thawed cells from 1-liter cultures were resuspended in 25 mL of 20 mM Na₂HPO₄, pH 7.2, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and one tablet Complete (Roche). Lysated cells were sonicated for four 1-min periods on ice followed by centrifugation at 18,000 rpm for 30 min. To precipitate heat-denatured proteins, the supernatants were placed in a water bath at 70°C for 20 min, at which time the samples had reached a temperature of ~55°C and then centrifuged at 18,000 rpm for 30 min. Supernatants were stored at −80°C.

**Purification**

Lti30 was purified by metal ion affinity chromatography and gel filtration. The supernatant from heat precipitation was diluted with 2 volumes of 20 mM Na₂HPO₄, pH 7.2, 1.88 M NaCl, and 1 mM phenylmethylsulfonyl fluoride. The sample was loaded on a 5-mL Hitrap IDA-Sepharos column (GE Healthcare) charged with 7 mL of 3 mg/mL CuSO₄. The column was equilibrated with 5 volumes of 20 mM Na₂HPO₄, pH 7.2, 1.0 M NaCl was used to equilibrate the column, and 40 volumes of this buffer was used to wash off unbound sample from the column. Fractions of 5 mL were collected for analysis throughout the run. Elution was performed with 2 M NH₄Cl in 20 mM Na₂HPO₄, pH 7.2, and 1.0 M NaCl in one step. The column was then equilibrated with 10 volumes of 20 mM Na₂HPO₄, pH 7.2, followed by elution of the copper with 10 mM EDTA in 20 mM Na₂HPO₄, at pH 7.2. Precipitation of protein was done with 80% (NH₄)₂SO₄, and protein was collected by centrifugation at 18,000 rpm for 30 min. Lti30 was resuspended in 2.5 mL of 50 mM glycine, pH 9.0, and desalted in the resuspension buffers on a PD-10 column (GE Healthcare). The proteins were loaded on an S-100 gel filtration column connected to an AKTA system, with a flow rate of 2.0 mL/min and absorbance read at 280 nm. Fractions of 2 mL were collected during the run. The purity was tested by SDS-PAGE gel electrophoresis (Bio-Rad). Protein quantification was measured with the bicinchoninic acid assay (Sigma-Aldrich).

**Lipids**

Phosphatidylcholine, PG, and phosphatidylserine were purchased from Avanti Polar Lipids with either DO or DM as fatty acids.

**Vesicle Preparation**

LUVs (100 nm) of DOPC, DOPG, and DOPS alone or DOPC mixed with either DOPG or DOPS (3:1 molar ratio) were prepared by the method of extrusion. The lipids were dissolved in chloroform, and lipid mixtures were dried under a gentle nitrogen flow and subsequently hydrated in buffer (50 mM glycine, pH 9.0, 10 mM phosphate buffer, pH 7.2, 5 mM MES, pH 6.3, or 10 mM KH₂PO₄ buffer, pH 4.3) and vortexed for 10 min. Five cycles of freeze/thaw in liquid nitrogen to reduce lamellarity followed, and the lipid solution was extruded 20 times through an extruder (Avanti) with a 0.1-mm pore size polycarbonate filter. DMPC:DMPG (3:1 molar ratio) liposomes were prepared as above from a stock solution of 9 mM, and before runs 3 mL liposome stock was extruded in an Avanti Mini Extruder (100-nm poly carbonate filter, repeated 21 times) and diluted in respective phosphate buffer to final concentration. For the pH titration experiments, 10 mM KH₂PO₄ and 10 mM K₂HPO₄ were mixed to obtain the desired pH and measured by a pH meter. The vesicles were then prepared as above in the respective phosphate buffer. No aggregation of vesicles in the absence of Lti30 could be detected at any pH (see Supplemental Figure 8) online.

**Dehydrin–Phospholipid Interaction Studies: Surface Plasmon Resonance**

Surface Plasmon resonance was performed on a Biacore3000 (GE Healthcare). This technique is used systematically to study various biomolecular interactions with both lipids and proteins (Besenica et al., 2006). In the Lti30 and phospholipid recognition studies, the phospholipid vesicles were immobilized on sensor chip surfaces, and the dehydrins served as the soluble analytes. The lipid binding L1 chip (Biacore) has four lipid binding surfaces corresponding to the flow cells. In an experiment, three of the surfaces are covered with different lipid vesicles and the fourth unmodified (lipid-free) flow cell served as reference and control.
The same dehydrin sample was run at the same time in all four cells. Figure 2 shows a typical result from such an experiment. All buffers were filtered (0.22 μM) and degassed prior to use. Typically, liposomes (0.05 to 2 mM) were diluted in 0.5 mM MES buffer and captured to saturation (5 min) across isolated flow cells at 2 μL/min. Unmodified (lipid-free) flow cells served as reference and control surfaces. Fresh liposomes were injected for each analyte to ensure that analysis was unaffected by previous injections. The flow system, except the sensor surface, was washed with 2:3 (v/v) isopropanol/50 mM NaOH after each liposome injection to minimize carryover from previous injections. A liposome injection time of 7.5 min at 2 μL/min was chosen to achieve a stable liposome surface. Liposomes were reproducibly immobilized to levels of ~7400 ± 90 RU for DOPC and ~6500 ± 160 RU for the mixtures of DOPC with negatively charged DO phosphate lipids. For DMPC, the immobilization level was ~6700 ± 70 RU and for the mixtures of DMPC with negatively charged DM phosphate lipids ~6200 ± 130 RU. The liposome binding levels were subtracted for baseline correction. Typically, 10 liposome injections were made per assay with relative standard deviations of 1.0 to 2.0%. After liposome binding, a 10-μL injection of 0.1 mg/mL BSA resulted in an increased signal of 39 ± 29 RU compared with 563 ± 46 RU for dextran matrix in the absence of lipid. This was because BSA binds strongly to the dextran matrix of the L1 sensor chips but weakly to lipid bilayers (Erb et al., 2000). Lti30 (10 μM) was injected at a flow rate of 0.2 mL/min. At the end of sample injection, running buffer was flowed over the sensor surface to facilitate dissociation of unbound protein. The response was monitored as a function of time (sensogram) at 25°C. All Biacore experiments were run in duplicate or triplicate to confirm reproducibility.

NMR Measurements

For NMR analysis, lipid films (as prepared above) were resuspended with appropriate protein solutions and pelleted to a highly viscous protein/lipid suspension. NMR measurements were performed using a 400 MHz Infinity spectrometer (Chemagnetics/Varian) equipped with a 4-mm double resonance probe. 31P magic angle spinning NMR spectra were acquired at 308K in single pulse experiments with simultaneous proton decoupling. Spectra were referenced using a DMPC standard at 308K (Lindström et al., 2005).

Dehydrin–Phospholipid Interaction Studies: DSC

Heat capacity profiles were recorded on a VP-DSC Micro Calorimeter. The scan module contained a first fast heating and cooling with 60°C/h (5 to 60°C) followed by a second heating rate of 2°C/h (5 to 45°C). Sample concentrations were 3 mM of lipids DMPC:DMPG (3:1 molar ratio) and 30 μM Lti30 (in 5 mM MES pH 6.3). For a lipid:protein molar ratio of 30:1, we used 0.9 mM DMPC:DMPG (3:1 molar ratio) and 30 μM Lti30. In the case of the phosphorylated Lti30 (12 μM), a lipid concentration of 1.2 mM DMPC:DMPG (3:1 molar ratio) was used, resulting in a molar ratio protein:lipid of 1:100. Samples were degassed and measured. Data analysis was performed by the origin software package.

Light Microscopy

For light microscopy analysis, a 1:100 Lti30-to-lipid molar ratio was used. Vesicles were incubated with or without Lti30 (14 μM) at room temperature, 60-μL samples were put on a glass slide, and three images per droplet were examined using an inverted Zeiss Axiovert 40 CFL microscope equipped with a digital Aciocam ICC1 camera at 100× magnification (lens, phase contrast 1-04; Zeiss).

Thylakoid Membrane Preparation

Forty grams of spinach (Spinacia oleracea) and 100 mL of 0.3 M Suc, 50 mM Na-phosphate, pH 7.4, and 5 mM MgCl2 was placed in a cold mixer and mixed for 5 × 10 s. The solution was filtered and centrifuged at 3000 rpm for 3 min. The pellet was suspended in 30 mL of 0.3 M Suc, 50 mM Na-phosphate, pH 7.4, and 5 mM MgCl2 and centrifuged at 4500 rpm for 5 min. The solution was homogenized in 30 mL 10 mM phosphate, pH 7.4, 5 mM MgCl2, and 5 mM NaCl and centrifuged at 4500 rpm for 5 min. The pellet was homogenized in 12 mL of 0.1 M Suc, 10 mM phosphate, pH 7.4, 5 mM MgCl2, and 5 mM NaCl.

Determination of Lti30 in Supernatant and Vesicle Pellet

Lti30 (0.15 mg/mL) was mixed with DOPC:DOPG (1:4 mM, 3:1 molar ratio) at pHs of 4.3, 6.3, 7.2, and 9.0. Samples were centrifuged at 12,000 rpm for 20 min. Ten microliters of the supernatant (original volume 40 μL) was mixed 1:1 with SDS-PAGE cocktail. The pellets were resuspended in 10 μL 50 mM glycine, pH 9.0, and mixed 1:1 with SDS-PAGE cocktail. Samples were boiled and 10 μL was loaded on a 15% SDS-PAGE gel (Bio-Rad). For the total protein concentration measurements, 0.2 mg/mL Lti30 was mixed with DOPC:DOPG (1:4 mM, 3:1 molar ratio) at a total volume of 100 μL at pHs of 4.3, 6.3, 7.2, and 9.0 (see above). Samples were centrifuged at 12,000 rpm for 20 min. Pellets were dissolved in 100 μL 50 mM glycine, pH 9.0, and protein concentration of both supernatant and pellet was measured with the BCA kit (Sigma-Aldrich).

Phosphorylation of Lti30

Lti30 (0.3 mg/mL) was phosphorylated with 4.76 units mL⁻¹ PKC from rat brain (Merck) in 1 mM ATP, 20 mM MgCl2, 4 mM CaCl2, 4 mM EGTA, and 10 mM Tris-HCl, pH 7.5, for 4 h at room temperature. Cor47 and Lti30 (both at 0.2 mg/mL) were phosphorylated with 5.4 units/mL CK1 (rat liver; Sigma-Aldrich) in 1 mM ATP, 50 mM KCl, 10 mM MgCl2, and 20 mM Tris-HCl, pH 7.5, for 4 h at room temperature. Phosphorylation status was tested with 32P isotope marking by adding 0.5 μL of 0.2 mM ATP32 to the reaction mix. Samples were run on SDS gels. Radioactivity on dried gels was detected with a FLA-3000g (Fuji Photo Film).

Proteolytic Digestion of Free and Vesicle-Bound Lti30

Vesicle-bound Lti30 was prepared by mixing 1 volume Lti30 (14 μM) with 100 volumes DOPC:DOPG vesicles (3:1) (1.4 mM) at pH 7.2 (50 mM MES). Free Lti30 was prepared by mixing 1 volume Lti30 (14 μM) with 100 volumes buffer at pH 7.2 (50 mM MES). To start the digestion, 0.3 μg trypsin from bovine pancreas (Sigma-Aldrich) was added to 200 μL of either vesicle-associated or free Lti30, yielding a final trypsin to Lti30 ratio of 1:200. Digestion was terminated at the designated time points by adding 1 μL phenylmethanesulfonyl fluoride (Sigma-Aldrich) to 10 μL sample. All samples were mixed with equal volumes SDS cocktail and heated for 5 min and then run on a 15% ready SDS-PAGE gel (Bio-Rad). Samples were also taken for microscopy analysis.

Absorbance and CD Analysis

Absorbance measurements were performed on an Ultrospec 3300 pro (Amersham) at 400 nm and at 25°C. Far-UV CD spectra were recorded on an Applied Photophysics spectrophotometer with a scan rate of 20 nm/ min at 0.2-nm resolution and 20 mdeg sensitivity. All samples were mixed 1 h before the CD run and centrifuged at 12,000g for 2 min before filling the 0.2-mm cuvette. Protein concentration was 1 mg/mL in 5 mM MES buffer at pH 6.3. All runs were performed at 25°C, unless otherwise stated. All CD spectra are presented as mean ellipticity per residue.

Prediction Program

NetphosK and ProtParam are freely available at the ExPASY Tools homepage.
Accession Number
Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession number P42758 (dehydrin Lti30).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. Binding of Lti30 and K-Segment to DOPC on Biacore.

Supplemental Figure 2. Differential Scanning Calorimetry Data of Lti30 and the Dehydrin Cor47 in the Presence of DMPC:DMPG LUVs (3:1 Molar Ratio) at L.P, 100:1 (Lti30 and Cor47), and 1:30 (Lti30).

Supplemental Figure 3. Lti30 and Calcein Vesicle Leakage Experiments.

Supplemental Figure 4. The K-Segments, with or without Flanking His Residues, Ability to Aggregate Lipid Vesicles.

Supplemental Figure 5. CD Spectra of Lti30 and Phosphorylated Lti30.

Supplemental Figure 6. Titrations of Lti30 and Phosphorylated Lti30 (Lti30P) into 1.4 mM DOPC:DOPG LUVs (3:1 Molar Ratio) at pH 7.2.

Supplemental Figure 7. Amino Acid Sequences of the Vesicle Binding Maize DHN1(YSK2) and the Nonvesicle Binding Soybean rGMDHN1 (YK).

Supplemental Figure 8. Light Microscopic Picture of DOPC:DOPG (1.4 mM, 3:1 Molar Ratio) LUVs at Different pH Showing No Aggregation at Any pH.

Supplemental Table 1. The 99 Different K-Segments of the Dehydrins in Table 1, Grouped According to the Pattern of their His Flanks.

Supplemental Table 2. Composition of K-Segments in the Dehydrins in Table 1.

ACKNOWLEDGMENTS
We thank Åke Wieslander and Mikael Oliveberg for helpful discussions. Financial support was given by the Magnus Bergvalls Stiftelse (P.H), the Lawski Foundation (S.K.E.), and the Swedish Research Council (G.G.).

Received March 11, 2011; revised May 5, 2011; accepted May 27, 2011; published June 10, 2011.

REFERENCES


koag, m.c., wilkens, s., fenton, r.d., resnik, j., vo, e., and close, t.j. (2009). the k-segment of maize dhn1 mediates binding to anionic phospholipid vesicles and concomitant structural changes. plant physiol. 150: 1503–1514.  
koopman, e.e., tieleman, d.p., testerink, c., munnik, t., rijkers, d.t., burger, k.n., and de kruijf, b. (2007). an electrostatic/hydrogen bond switch as the basis for the specific interaction of phosphatidic acid with proteins. j. biol. chem. 282: 11356–11364.  
lee, s.a., eyeson, r., cheever, m.l., geng, j., verkhusha, v.v., burd, c., overduin, m., and kutateladze, t.g. (2005). targeting of the fyye domain to endosomal membranes is regulated by a histidine switch. proc. natl. acad. sci. usa 102: 13052–13057.  
Tunable Membrane Binding of the Intrinsically Disordered Dehydrin Lti30, a Cold-Induced Plant Stress Protein
Sylvia K. Eriksson, Michael Kutzer, Jan Procek, Gerhard Gröbner and Pia Harryson
Plant Cell 2011;23;2391-2404; originally published online June 10, 2011;
DOI 10.1105/tpc.111.085183

This information is current as of October 28, 2017

Supplemental Data /content/suppl/2011/05/31/tpc.111.085183.DC1.html
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