Determination of structural requirements and probable regulatory effectors for membrane association of maize sucrose synthase 1.

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Sucrose synthase (SUS) cleaves sucrose to form UDP-glucose and fructose, and exists in soluble (s-SUS) and membrane-associated (m-SUS) forms, with the latter proposed to channel UDP-glucose to the cellulose synthase complex on the plasma membrane of plant cells during synthesis of cellulose. However, the structural features responsible for membrane localization and the mechanisms regulating its dual intracellular localization are unknown. The *Zea mays* (L.) SUS1 isoform is likely to have the intrinsic ability to interact directly with membranes because we show: (i) partial membrane localization when expressed in *Escherichia coli*, and (ii) binding to carbonate-stripped plant microsomes *in vitro*. We have undertaken mutational analyses (truncations and Ala substitutions) and *in vitro* microsome binding assays with the SUS1 protein to define intrinsic membrane-binding regions and potential regulatory factors that could be provided by cellular microenvironment. The results suggest that two regions of SUS1 contribute to membrane affinity: (i) the amino-terminal non-catalytic domain and (ii) a region with sequence similarity to the C-terminal PH (pleckstrin homology) domain of human pleckstrin. Ala substitutions within the PH-like domain of SUS1 reduced membrane association in *E. coli* and with plant microsomes *in vitro* without reducing enzymatic activity. Microsomal association of wild type SUS1 displayed cooperativity with SUS1 protein concentration and was stimulated by both lowering the pH and adding sucrose. These studies offer insight into the molecular level regulation of SUS1 localization and its participation in carbon partitioning in plants. Moreover, transgenics with active SUS mutants altered in membrane affinity may be of technological utility.
INTRODUCTION

Glycosyltransferases (GTases) constitute a large group of enzymes involved in the biosynthesis of carbohydrates and glycoconjugates in prokaryotes and eukaryotes. The diversity of compounds synthesized by these enzymes and their varied intracellular locations implicates them in storage, structural and signaling functions. Currently, 77 families of GTases are recognized (Coutinho et al., 2003; http://afmb.cnrs-mrs.fr/CAZY/). Although these families possess very little sequence homology, the enzymes within a given GTase family are expected to fold similarly into a GT-A or GT-B type structure. These enzymes are further classified as inverting or retaining GTases depending upon whether the products formed invert or maintain, respectively, the stereochemistry at the C1 position of the donor sugar.

In bacteria and fungi, numerous GTases have been implicated in the biosynthesis of extracellular or membrane-localized compounds. For instance, the *Streptococcus pneumoniae* enzyme WciS is a retaining GTase included in family GT4 that is involved in capsular polysaccharide biosynthesis. This multimeric protein is membrane-associated although it lacks any predicted transmembrane sequences (Saksouk et al., 2005). Similarly, the *Escherichia coli* MurG enzyme is an inverting GTase with a GT-B fold included in family GT28 that is involved in peptidoglycan (bacterial cell wall) biosynthesis. This GTase as well is only moderately hydrophobic and lacks a membrane-spanning domain, yet it is still associated with the cytoplasmic surface of the inner membrane (Ha et al., 2000). The *Pichia pastoris* Ugt51 enzyme is an inverting GTase included in family GT1 that produces sterol glucosides. This protein contains two lipid-binding modules, a GRAM- and a PH-domain, and is associated with the micropexophagic membrane apparatus \textit{in vivo} (Oku et al., 2003). Bacterial α-monoglucosyldiacylglycerol synthases are included in family GT4 and are membrane-associated GTases involved in modulating bilayer and lipid surface properties (Berg et al., 2001; Edman et al., 2003). These proteins also lack transmembrane domains but have predicted structural and sequence similarities to *E. coli* MurG that may impart membrane affinity.

Cellulose is one of the most abundant carbon reserves in nature, constituting a large portion of the dry matter content of grasses and wood. Consistent with bacterial extracellular matrix deposition, plants utilize numerous GTases during cell wall biosynthesis (Buckeridge et al., 1999; Egelund et al., 2004; Charnock et al., 2001). Cellulose synthase is itself an inverting
GTase belonging to family GT2 that synthesizes the β-1,4-linked glucose polymer cellulose from UDP-glucose. Moreover, a membrane-bound GTase (UDP-glucose:sterol glucosyltransferase) has been implicated in the production of a sitosterol-β-glucoside primer that initiates cellulose polymerization (Peng et al., 2002). In developing cotton ovules during the peak of secondary cell wall formation, cellulose synthesis can consume roughly 80% of all imported carbon (Haigler et al., 2001). In most plant species, the cellulose biosynthetic pathway is supported by carbon skeletons obtained from imported sucrose.

Sucrose synthase (SUS) enzymes are also classified as retaining GTases belonging to family GT4, and are thought to adopt a GT-B type fold similar to E. coli MurG (MacGregor, 2002). SUS catalyzes a reaction that converts sucrose and UDP into UDP-glucose and fructose (Tsai, 1974), and can exist in association with the plasma membrane (Amor et al., 1995; Carlson and Chourey, 1996; Winter et al., 1997; Sturm et al., 1999; Haigler et al., 2001; Komina et al., 2002). Plasma membrane-associated SUS is postulated to channel its product, UDP-glucose, towards the synthesis of cellulose and callose (Amor et al., 1995; Winter and Huber, 2000; Haigler et al., 2001; Koch, 2004). The involvement of SUS in cellulose deposition is supported by expression and localization of protein and activity (Amor et al., 1995; Ruan et al., 1997; Kordel and Kutschera, 2000; Salnikov et al., 2001; Uggla et al., 2001; Kutschera and Heiderich, 2002; Salnikov et al., 2003; Albrecht and Mustroph, 2003) in cotton (Gossypium hirsutum L.), Zinnia elegans (L.), wheat (Triticum aestivum L.), pea (Pisum sativum L.), sunflower (Helianthus annuus L.) and pine (Pinus sylvestris). Moreover, the importance of SUS in this process is evidenced by genetic over-expression (Konishi et al., 2004) and suppression or mutation (Chourey et al., 1998; Sturm and Tang, 1999; Ruan et al., 2003). It is therefore well established that SUS can exist on membranes and channel UDP-glucose to cellulose biosynthesis. However, it is unclear how this mainly soluble enzyme is redistributed to the plasma membrane to fulfill this function.

In maize, SUS is a tetrameric enzyme composed of subunits encoded by the Sh1, Sus1 and Sus2 loci, which encode the SUS-SH1, SUS1 and SUS2 proteins, respectively (http://www.maizegdb.org/cgi-bin/displaygprecord.cgi?id=13861). Both the SUS-SH1 and SUS1 proteins associate with the plasma membrane in maize endosperm (Carlson and Chourey, 1996), whereas the intracellular distribution of SUS2 (previously known as SUS3) in maize is currently being determined (Duncan et al., 2005). SUS is peripherally associated with maize leaf
microsomes, and the abundance of membrane-associated SUS (m-SUS) is variable along the longitudinal axis of developing maize leaves (Hardin et al., 2004). Therefore, it is unlikely that m-SUS represents a distinct gene product but rather that individual SUS isoforms can exist in multiple intracellular locations. At maximum, m-SUS constituted about 9-10 % of total cellular SUS in maize leaves (Winter et al., 1997; Hardin et al., 2004) and 3-14 % in endosperms (Carlson and Chourey, 1996), whereas it represented 25-50 % of total SUS in cotton fiber membranes and carrot protoplasts (Amor et al., 1995; Sturm et al., 1999). These observations strongly suggest that the association of SUS with the plasma membrane is a controlled and regulated process in plants.

In this manuscript, we identify a region of SUS that possesses significant sequence homology to the C-terminal PH (pleckstrin homology) domain of human pleckstrin. Since PH-domains are known to impart polyphosphoinositide and/or membrane localization to about 30 % of the proteins that contain them (Lemmon et al., 2002; Yu et al., 2004; Edlich et al., 2005), we investigated the involvement of this region in SUS1 membrane binding. We demonstrate that SUS1 has an intrinsic ability to interact directly with membranes, and that this ability is due, in part, to the identified PH-like domain. In addition, we demonstrate that the N-terminal non-catalytic region also contributes to membrane binding. The presence of sucrose and low pH were identified as factors that promote partitioning of SUS1 to membranes. These experiments provide a foundation for guiding the rational creation of mutant SUS transgenics that possess altered membrane localization, and to potentially control carbon allocation to structural compounds in plants.

RESULTS

Structural Analyses of SUS Substantiate its Assignment as a GTase and Reveal Regions Potentially Involved in Membrane Binding

Bioinformatic analyses of the SUS1 primary sequence revealed the presence of a region between amino acids 360 and 457 with striking sequence similarity to the C-terminal PH-domain contained within human pleckstrin (Fig. 1A). The alignment of these regions in SUS1 and human pleckstrin required only a single amino acid gap over this 98 amino acid stretch and possessed 34 % similarity. The C-terminal PH-domain of human pleckstrin binds phosphatidylinositol-(3,4)-bisphosphate (Edlich et al., 2005), making the related region of SUS a possible contributor to membrane binding. This PH-like domain of SUS1 is also highly conserved in the two other
known maize SUS isoforms (≥74% identity among the SUS isoforms) (Fig. 1A). However, the secondary structures predicted by the Jpred consensus method (http://www.compbio.dundee.ac.uk/~www-jpred/) differ significantly between SUS and the PH-domain of human pleckstrin (Fig. 1A). SUS possesses an alternating β strand-α helix arrangement, whereas the PH-domain of pleckstrin consists of sequential β strands bordered at both termini by α helices. Therefore, an identical tertiary structure of these two regions is unlikely.

The secondary structure of SUS has recently been modeled onto a GTase GT-B type fold (MacGregor, 2002). Indeed, our own analyses (Fig. 1B) demonstrated that the secondary structure of amino acids 281-769 of maize SUS1 predicted by Jpred was consistent with this putative catalytic structure, although the α2 and α5 helixes seemed to be replaced by β strands. Therefore, it is presumed that the amino-terminal 280 amino acids and the carboxyl-terminal 47 amino acids represent non-catalytic regions of SUS (Figure 1B). Moreover, amino acids 281-780 of SUS1 could be modeled onto the structures of Agrobacterium tumafaciens glycogen synthase (SCOP code c1rzvA), and E. coli MurG (SCOP code d1f0ka) by the Protein Homology/analogy Recognition Engine (phyre) server (http://www.sbg.bio.ic.ac.uk/~phyre/) with 100% precision and E-values less than 1.4e-06 even though sequence identity was less than 13%. Notably, both of these enzymes are known to posses a GT-B type fold (Ha et al., 2000; Buschiazzo et al., 2004). The predicted GT-B type structure represents the entire catalytic domain of SUS1 (amino acids 281-780) and consists of a bilobed configuration with large amino and carboxyl domains joined by a helical linker (Fig. 1B). The N-terminal lobe is thought to possess the acceptor binding sites, while the C-terminal lobe provides the donor binding sites, as well as the putative catalytic glutamate residues within the Ex7E motif (E678E686 in SUS1) characteristic of retaining GTases. The identified PH-like domain of SUS (Fig. 1A) falls within the N-terminal catalytic lobe between β2 and β6 (Fig. 1B). The SUS β3 segment and the β3/α3 loop sequence are enriched in hydrophobic residues and well conserved among SUS isoforms. Notably, it is amino acids within this region of the C-terminal human pleckstrin PH-domain which contact phosphatidylinositol-(3,4)-bisphosphate and confer specificity (Isakoff et al., 1998; Edlich et al., 2005). The presence of a proline (P371) and different secondary structural elements in this region of SUS may alter this property in its PH-like domain. Both termini of SUS polypeptides contain additional sequences outside of the catalytic domain, the largest (280 amino acids) being...
at the N-terminus (Fig. 1B). Acceptable models for these non-catalytic regions of SUS1 could not be identified through the Protein Homology/analogy Recognition Engine (phyre) server. However, the predicted tertiary structure of the SUS catalytic domain allows for comparisons with other known GTase structures and membrane binding regions. Significantly, the PH-like domain of SUS occupies an identical region of the GTase molecule (β3-α5) as does the proposed membrane association site of MurG (β3-α5) (Ha et al., 2000). This region of the MurG structure is occupied by a surface exposed hydrophobic patch surrounded by basic residues; similar amino acid types are found in SUS between β3 and α3 (Fig. 1A).

Analyses of SUS1 Truncation Mutants Reveals that Membrane Binding Involves the Amino-Terminal Non-Catalytic Domain

To address the involvement of the PH-like domain of SUS in membrane binding, we produced N-terminal MBP-tagged truncation mutants of the SUS1 polypeptide (Figs. 1B and 2) to isolate this region. The large MBP tag was selected for its recognized ability to maintain recombinant protein solubility in *E. coli*. As reported earlier, we have found that a significant portion of 6His-tagged SUS1 recombinant protein was associated with bacterial membranes when expressed in *E. coli* (Hardin et al., 2004). When the various protein truncation mutants of MBP-SUS1 were expressed and the bacterial extracts fractionated into soluble (100K supers) and insoluble (100K pellets) components, even the smallest construct (T362stop) that contained the N-terminal non-catalytic domain but lacked the PH-like domain partitioned strongly to the membrane fraction (Fig. 2). However, both full length MBP-SUS1 and the Y511stop proteins, which contain the entire N-terminal catalytic lobe and the PH-like region, displayed a slightly greater tendency to partition with the insoluble material.

The 100K soluble fraction of these MBP-SUS1 recombinant proteins was analyzed by size-exclusion chromatography to determine whether the oligomeric structure of SUS1 was affected by truncation, as this may be relevant to its observed partitioning and membrane binding behavior. MBP itself eluted as a monomeric protein (Fig. 3), whereas all of the MBP-SUS1 proteins eluted as dimers. This observation differs from the typically tetrameric form usually observed for native SUS, and may be related to the presence of an MBP tag. However, it clearly validated that all the MBP-SUS1 recombinant proteins were identical in this regard and therefore oligomeric structure would not be the source of any potential differences. It should also be stated
that equivalency in analysis by size exclusion chromatography (Fig. 3) does not imply that these proteins are equivalently folded or structured as predicted (Fig. 1B).

Although the relative intracellular partitioning of the MBP-SUS1 recombinant proteins in *E. coli* (Fig. 2) suggested that the full length protein contained additional determinants for segregation into the 100K pellet, it did not necessarily show that this partitioning behavior reflected membrane binding. Therefore, as a rigorous demonstration of membrane association that did not rely on co-sedimentation, we fractionated the 100K pellets by sucrose gradient flotation (Fig. 4). Under these conditions membranes and their associated proteins floated to the top of the gradient (Ahola et al., 1999), while inclusion bodies and soluble protein remained in the bottom of the gradient. Full length MBP-SUS1 protein obtained from the 100K supernatant (soluble) fraction did not float in these assays (Fig. 4), whereas about 35% of the full length protein recovered from the 100K pellet was shown to be unequivocally membrane associated as it floated to the top of these gradients. Between 15-25% of the other MBP-SUS1 truncation proteins were also membrane associated (Fig. 4). These results demonstrated that even the smallest recombinant protein (T362stop), which includes the N-terminal non-catalytic region but not the PH-like domain, contained determinants that provided association with bacterial membranes.

To test the involvement of these SUS1 regions in binding to plant-derived membranes, we mixed the various MBP-SUS1 recombinant proteins with microsomes obtained from the basal 4-16 cm region of elongating maize leaves and subsequently floated the membranes, and any protein that had bound to them, to the top of sucrose gradients (Fig. 5). Since SUS is known to interact with a variety of different membrane types in maize (Carlson and Chourey, 1996; Winter et al., 1998; Buckeridge et al., 1999; Etxeberria and Gonzalez, 2003) we purposefully prepared unfractionated total microsomes containing all these membrane types for these studies. The fact that the free MBP in these MBP-SUS1 recombinant protein samples did not float demonstrated the selectivity that this assay provided for membrane association. Under these conditions, the Y511stop protein, which contains the N-terminal non-catalytic lobe and the N-terminal catalytic lobe with its PH-like domain, associated with plant microsomes slightly better than all other recombinant proteins (Fig. 5). Therefore the PH-like domain of SUS1 might be contributing determinants that are of significance for binding to plant membranes, in addition to the determinants within the N-terminal non-catalytic region, but our experiments cannot discern
the origin of the membrane being bound in these plant-derived microsomes. The most striking difference between bacterial (Fig. 4) and plant (Fig. 5) membrane association assays was observed for the full length MBP-SUS1 protein. Its binding to plant microsomes was relatively weaker than to *E. coli* membranes, suggesting that differences in the source of the membranes were likely to contribute to SUS1 affinity. This assumption was supported by similar membrane binding experiments in which microsomes derived from two different regions of the elongating maize leaf were used (Fig. 6). No binding of the full length MBP-SUS1 protein to microsomes derived from the basal (4-16 cm) region was again observed, whereas it interacted well with microsomes derived from the apical (24-36 cm) region. Conversely, the T362stop protein interacted best with the basal microsome sample. These results suggested that the two regions of SUS1 implicated in membrane binding (i.e., the N-terminal non-catalytic domain and the PH-like domain) potentially provide affinity to membranes that may differ in lipid or lipid headgroup types. Moreover, treatment of these microsomes with carbonate, to remove peripherally-associated proteins that SUS1 may have affinity for, eliminated > 50% of the protein content (data not shown) but did not significantly affect the binding of either the T362stop or full length MBP-SUS1 proteins (Fig. 6). Although integral membrane proteins remain in these plant microsomes, these carbonate washing results and the observation that SUS1 will bind to bacterial membranes (Fig. 4) that contain a membrane protein composition dissimilar to plant microsomes, supports the supposition that SUS1 can interact directly with the lipid bilayer rather than another protein to confer membrane localization.

**Site Directed Mutants in the SUS1 PH-like Domain Reveal its Contribution to Membrane Binding**

Individual amino acids within the identified PH-like domain of SUS1 were converted to Ala (Fig. 1) in order to determine their contribution to membrane affinity. All Ala-substituted mutant proteins were evaluated for intracellular distribution in *E. coli* but behaved very similar to wild type (~ 70 % partitioned to 100K pellet; data not shown). However, when the amount of these recombinant proteins that bound to bacterial membranes was evaluated by sucrose gradient flotation of the 100K pellet, the substitution of certain hydrophobic and acidic residues with Ala significantly reduced the membrane binding ability of SUS1 (Fig. 7A). In particular, binding to *E. coli* membranes was reduced by 30-50 % in the I367A, E387A and W389A recombinant mutant proteins. Conversely, substitution of the basic residue at position 373 with Ala (R373A)
marginally (~10%) increased SUS1 binding. These results not only enforced the conclusion that the SUS1 PH-like domain positively contributes to membrane affinity, but also suggested that binding may involve both hydrophobic and electrostatic interactions. These mutated residues occur primarily in a loop between β3 and α3 and do not alter the predicted secondary structure of the region (Fig. 1A; data not shown), implicating the importance of the individual amino acid itself to membrane binding. Significantly, these mutations did not interfere with the enzymatic (sucrose cleavage) activity of SUS1 (Fig. 7B), as all recombinant proteins maintained essentially the same activity as wild type.

Selected Ala-substituted mutant proteins were tested for binding to plant membranes by mixing them with microsomes obtained from the apical 24-36 cm region of elongating maize leaves and flotation in sucrose gradients. Similar to the binding observed for these proteins to bacterial membranes (Fig. 7A), mean values for binding to plant microsomes were reduced in the I367A and W389A mutants by about 50% (Fig. 8). Binding to plant microsomes was also marginally higher again in the R373A mutant protein in comparison to wild type.

**Sucrose and Low pH are Identified as Potential Physiological Effectors of SUS1 Membrane Binding**

The effect of different sugars on the interaction of SUS1 with membranes was evaluated by mixing the full length MBP-SUS1 protein with carbonate-washed microsomes obtained from the apical 24-36 cm region of elongating maize leaves followed by pelleting and washing to remove unbound protein (Fig. 9). This type of assay produced a higher level of background due to tube absorption but was necessitated by the need to perform the binding reactions under reduced sugar levels, which cannot be done in the sucrose gradient-dependent flotation assays that completely lacked background. However, these experiments clearly revealed that sucrose was important for the ability of SUS1 to bind plant microsomes (Fig. 9), as binding was reduced about 95% at low sucrose concentrations. It is important to note that in all other experiments sucrose was always maintained at 150mM or greater. Glucose and maltose at equimolar amounts to sucrose mimicked this effect in these assays suggesting the involvement of the glucosyl unit of sucrose.

When the association of full length MBP-SUS1 to apical (24-36 cm) microsomes was tested at pH 7.5 versus pH 5.5 by flotation (Fig. 10A), a dramatic stimulation (> 6-fold) in membrane binding was observed at the lower pH. In similar flotation assays performed with
increasing amounts of MBP-SUS1, the binding of SUS1 to carbonate-washed apical microsomes at pH 5.5 was sigmoidal (Fig. 10B). Binding was not apparent at MBP-SUS1 concentrations less than 140 nmol µl⁻¹ and increased sharply at 180 nmol µl⁻¹, suggestive of a cooperativity affect of SUS1 concentration on binding. This effect could be due to the presence of the MBP-tag, but cannot be a source of variability in previous experiments because protein concentration within an experiment was constant and above the binding threshold. A further doubling of MBP-SUS1 concentration (360 nmol µl⁻¹) did not double the amount bound indicating that membrane binding is saturable (Fig. 10B).

DISCUSSION

The biosynthesis of cellulose is a universal aspect of plant growth that consumes a significant amount of photosynthetically fixed carbon. An emerging feature of carbon partitioning towards this extracellular sink involves the channeling of sucrose-derived UDP-glucose to the cellulose synthase complex by m-SUS (Amor et al., 1995; Winter and Huber, 2000; Haigler et al., 2001; Koch, 2004). However, we do not currently understand how m-SUS abundance is regulated, whether m-SUS interacts with the plasma membrane directly or complexes with other membrane proteins (e.g., CesA), and what regions of the SUS molecule are involved in membrane localization. In this study, we have addressed the protein structural requirements that allow the soluble enzyme SUS to interact with membranes. Specifically we focused on an approximately 100-amino-acid region of SUS that we identified as having significant sequence homology to the C-terminal PH (pleckstrin homology) domain of human pleckstrin (Fig. 1A). We conclude that: (i) SUS has an inherent affinity for membranes (Figs. 4, 6, 7) and therefore does not necessarily need to co-localize with another protein for membrane localization; (ii) the interaction of SUS with membranes involves both the N-terminal non-catalytic region (residues 1-362) and the PH-like domain (residues 360-457) (Figs. 5, 8); and (iii) factors that influence the binding of SUS to membranes include pH, membrane source and the concentrations of both SUS protein and sucrose (Figs. 6, 9, 10). We do not suggest that the intrinsic ability of SUS to bind membranes is exclusively responsible for dictating membrane localization in vivo, and cannot exclude the possibility that the interaction of SUS1 with plant microsomes in vitro reflects affinity for an integral membrane protein. Additional positive and negative influences on m-SUS may be supplied by localized protein:protein interactions that have been demonstrated or suggested to exist (Winter et al., 1998; Etxeberria and Gonzalez,
However an inherent affinity for membranes may partially explain the localization of SUS on membrane systems other than the plasma membrane, such as the tonoplast (Exteberria and Gonzalez, 2003), Golgi (Buckeridge et al., 1999) and peribacteroid membrane (Weinkoop and Saalbach, 2003) that are not involved in cellulose synthesis and are certain to differ in their membrane protein composition.

**The Sequence Determinants Identified for SUS Membrane Affinity Have Functional Counterparts in Various Proteins**

In GTases that obviously lack transmembrane domains, but are nonetheless found in association with membranes, sparse experimental data exists to suggest the regions involved in this ability. Our analyses of the interaction of the SUS1 GTase with bacterial membranes *in vivo* (Figs. 4, 7) and plant microsomes *in vitro* (Figs. 5, 6, 8, 9, 10) identified two regions involved in binding ability, namely the N-terminal 362 amino acids and the PH-like domain (amino acids 360-457) (Fig. 1A). The PH-like domain was the focus of the present study, and the determinants of membrane binding within the N-terminal domain are currently under investigation. The demonstration that multiple regions are involved in membrane affinity is rather common, particularly for PH-domain containing proteins (Lemmon et al., 2002; Oku et al., 2003; Yu et al., 2004).

The N-terminal 362 amino acids of SUS1 includes the entire non-catalytic domain (Fig. 1B) and a small portion of the catalytic domain. No structural templates for this region of SUS1 could be identified, and it lacks significant homology to other proteins known to interact with membranes. The entire SUS1 protein is not hydrophobic, possessing a grand average of hydrophobicity (GRAVY) score of -0.282, where higher positive values indicate greater hydrophobicity. Moreover, the N-terminal non-catalytic domain is also not hydrophobic (GRAVY score -0.353). However as pointed out earlier, transmembrane prediction algorithms identified a region (FLGTIPMVFNVVILSPHGFA) of the SUS1 sequence between amino acids 274-294 with high hydrophobicity (GRAVY score 1.324) (Carlson and Chourey, 1996; Winter and Huber, 2000). This hydrophobic domain of SUS1 is conserved in the other two maize SUS isoforms and dicot SUS sequences, and is included within the smallest truncation mutant (T362stop) that we demonstrate to have membrane binding affinity. It is clear that this hydrophobic domain does not function as a transmembrane helix because SUS is peripherally
associated with both microsomal and tonoplast membranes (Exteberria and Gonzalez, 2003; Hardin et al., 2004), and the core of this hydrophobic domain (VFNVVILS) constitutes the predicted β1 strand of the N-terminal catalytic domain (Fig. 1B). However, the preceding loop (FLGTIPM) is likely to be surface exposed and could contribute a hydrophobic determinant (GRAVY score 1.471) to membrane binding. This hydrophobic domain is only one possible contributor to membrane affinity within the N-terminal non-catalytic domain and does not rule out additional regions or modifications that influence m-SUS abundance. In particular, SUS1 is known to have a peptide S-thiolation site (Rohrig et al., 2004) and two sites of phosphorylation (Hardin et al., 2004) within the N-terminal non-catalytic domain, and phosphorylation has been shown to influence the binding of SUS to certain membrane types (Hardin et al., 2004).

The determinant of SUS1 membrane binding identified in the present study is the PH-like domain (amino acids 360-457) (Fig. 1A), which resides between the predicted N-terminal β3 strand and the α5 helix (Fig. 1B). Although this region of SUS has significant sequence homology to the C-terminal PH-domain of human pleckstrin, it is unlikely to adopt a similar overall structural fold (Fig. 1A). Therefore, GTase structures may provide a better structural template to evaluate this region. Significantly, the MurG crystal structure (Ha et al., 2000) demonstrates that many of the hydrophobic and basic residues within the same region (β3-α5) occupied by the SUS PH-like domain fold to form a surface exposed patch in MurG that has been suggested to constitute the membrane association site (Ha et al., 2000). Since SUS1 can easily be threaded onto the atomic coordinates of the solved MurG structure by the Protein Homology/analogy Recognition Engine (phyre) server, it is likely that the hydrophobic residues within the SUS1 PH-like domain form a similar hydrophobic patch. Further studies have demonstrated that the MurG protein has affinity for negatively charged lipids (i.e., cardiolipin) (van den Brink-van der Laan et al., 2003). This suggests that the SUS1 PH-like domain may confer affinity to anionic lipids, and indeed the loop sequence between β3 and α3 (ILRVFPRTENIVRKYFVWPYL) has a pI of 10.83. In fact, effects on membrane binding were demonstrated by mutation of specific residues (underlined in the preceding sequence) within this region (Figs. 7, 8). The predicted secondary structure of this region (Fig. 1A) is not altered by these Ala substitutions (data not shown), implicating a contribution of the individual amino acids themselves. Since the substitution of hydrophobic residues (I367 and W389) and an acidic residue (E387) with Ala decreased binding, whereas the basic residue
mutation R373A slightly increased binding, presumably the position specific contributions of hydrophobicity and negative charge influence SUS membrane affinity. Two of the residues (I367 and R373) we identified as contributing to SUS membrane binding are conserved (L251 and R257) in pleckstrin and have been identified as important for phosphatidylinositol-(3,4)-bisphosphate interaction (Isakoff et al., 1998; Edlich et al., 2005).

Interactions of Variables is Likely to Control the Abundance of m-SUS

Despite the fact that certainly some of the regulation of SUS abundance occurs at the transcriptional level (Winter and Huber, 2000; Koch, 2004), we propose that much of the regulation of m-SUS abundance occurs at the protein level via the targeting of soluble SUS (s-SUS) protein onto membranes. The relative abundance of m-SUS can vary dramatically (3-50 % of total) between plant species and organs (Amor et al., 1995; Carlson and Chourey, 1996; Winter et al., 1997; Sturm et al., 1999) and is developmentally or conditionally variable within a given organ (Amor et al., 1995; Winter et al., 1997; Subbaiah and Sachs, 2001). In maize leaves, the abundance of m-SUS is developmentally variable and not a fixed percentage of s-SUS (Hardin et al., 2004), demonstrating that m-SUS protein abundance is regulated by factors other than SUS abundance within the soluble phase.

In this report, we identify several factors that potentially influence the abundance of m-SUS in vivo including: pH, membrane source and the concentrations of both SUS protein and sucrose (Figs. 6, 9, 10). Effectors such as Ca$^{2+}$ and phosphorylation also influence m-SUS abundance directly (Winter et al., 1997; Haigler et al., 2001; Hardin et al., 2004), and salt and inorganic N stresses preferentially reduce m-SUS abundance in nodules (Komina et al., 2002). Lowering the pH from 7.5 to 5.5 enhanced the binding of SUS1 to microsomes (Fig. 10A). The pH optimum for the sucrose cleavage activity of SUS is acidic (pH 6.0-6.5) whereas sucrose synthetic activity is maximal at alkaline pH (8.0-8.8) (Tsai, 1974), suggesting that pH directly influences the conformation and enzymatic activities of SUS. Low pH (5.5) also reduces the helical conformation of at least the extreme N-terminus of SUS1 (Hardin et al., 2004). Coincident with these low pH-promoted conformational changes could be the exposure of membrane binding determinants.

The diversion of carbon towards cellulose biosynthesis rather than core metabolic requirements has been suggested to occur only when conditions are nearly optimal (Haigler et al., 2001). A component of establishing a near optimal condition for cellulose synthesis would be
provision of a sufficient supply of sucrose. In our experiments, the presence of higher levels (154 mM) of sucrose was nearly essential for SUS1 to bind leaf microsomes (Fig. 9). These effects on m-SUS binding occurred within the reported range for physiological, cytosolic sucrose concentrations (approximately 20-200 mM; Gerhardt et al., 1987; Winter et al., 1994), and was seemingly a direct effect on SUS itself. It has been shown that sucrose effects the conformation of SUS directly, but curiously it decreased the overall surface hydrophobicity (Winter et al., 1997). Hypothetically, SUS could be part of a sucrose-sensing mechanism for the carbohydrate level in sink tissues (Winter and Huber, 2000). These data also suggest the potential for m-SUS to localize in close proximity to sucrose:proton co-transporters, as locally the concentration of sucrose would be high and the pH low. Both of these conditions (Figs. 9, 10A) would be expected to promote the membrane binding of SUS in the vicinity of these transporters. The existence of acidic pH microdomains has been experimentally demonstrated (Schwiening and Willoughby, 2002), and data to suggest that SUS:sucrose transporter complexes exist has been provided (Etxeberria and Gonzalez, 2003).

SUS is known to interact with a variety of different membrane types and, while beyond the scope of this manuscript, a logical next level of evaluation involves determining the contribution of these sequence determinants and effectors on the binding of SUS to individual membrane types. Additionally, we are actively pursuing: (i) the creation of multiple and additional mutations within the N-terminal non-catalytic and PH-like domains, and (ii) the production of transient and stable transgenic plants to assess the in vivo significance of altered SUS membrane affinity on cellulose deposition and carbon utilization.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Maize (Zea mays L. cv Pioneer 3183 or inbred line B73) plants were grown in a soil mixture in a greenhouse and fertilized three times weekly with a modified Hoagland’s solution. Temperature was controlled by an Argus control system (White Rock, British Columbia, Canada) and supplemental lighting was provided by 1000W metal halide lamps. Immature leaves from the top of 4 to 8 week old plants were harvested, twelve cm segments were removed from the elongating, basal (4 to 16-cm) or apical (24 to 36-cm) regions of the leaves, immediately frozen in liquid nitrogen and stored at –80°C.

Preparation of Maize Leaf Microsomes
Frozen maize leaf was ground in 2 volumes of protein extraction buffer (100 mM Mops pH 7.5, 10 mM DTT, 5 mM EDTA, 5 mM EGTA, 20 mM NaF, 0.5 µM microcystin-LR, 1 mM AEBSF, 1 mM benzamidine, 5 mM caproic acid, 2 µM N-(trans-Epoxysuccinyl)-leucine-4-guanidinobutylamide (E64), 10 µM Carbobenzoxy-leucyl-leucyl-leucinal (MG132) (CalBiochem, San Diego, CA, USA), 0.05 g g⁻¹ FW polyvinylpolypyrrolidone, and 0.33 M sucrose. Clarified extracts were produced by filtration through Miracloth (CalBiochem, San Diego, CA, USA) and two sequential centrifugations at 9,500g and 4°C for 20 min. The microsomal pellet was recovered at 100,000g for 1 h at 4°C, then salt- and Brij58-washed to invert vesicles and release entrapped protein (Johansson et al., 1995) by resuspension in 50 mM Mops pH 7.5, 5 mM DTT, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 0.1 µM microcystin-LR, 1 mM benzamidine, 5 mM caproic acid, 2 µM E64, 0.25M sucrose, 150 mM NaCl and 0.05 % (w/v) Brij 58. The membrane fraction was pelleted at 100,000g for 1 h at 4°C and resuspended on ice in 4-10 % of the original volume in 50 mM Mops pH 7.5, 1 mM DTT, 0.1 mM EDTA, 5 mM NaF, 0.5 mM benzamidine, 2.5 mM caproic acid, 1 µM E64, 0.25M sucrose. Aliquots of membranes were treated with carbonate buffer (100 mM Na₂CO₃ pH 11.5, 1 mM DTT, 0.1 mM EDTA, 5 mM NaF, 0.5 mM benzamidine, 2.5 mM caproic acid, 1 µM E64 and 0.25 M sucrose for 30 min on ice (Fujiki et al., 1982) then recovered at 145,000g for 1 h at 4°C and resuspended as above.

**Production of Mutant SUS1 Recombinants and Proteins**

A maize SUS1 cDNA (GI #514945) in the pRSETB vector was used to construct sequencing-verified, site-directed mutants (Ala substitutions) with the QuikChange XL Kit as recommended by Stratagene (La Jolla, CA, USA). Subcloning of a T4 DNA polymerase-blunt ended PvuI, BamHI SUS1 fragment into a T4 DNA polymerase-blunt ended PstI, BamHI digested pMAL-c2x vector (New England Biolabs, Beverly, MA, USA) was used to produce the full-length MBP-SUS1 construct. Sequencing-verified, site-directed mutants (stop codon insertions) were produced as above. All constructs were transformed into *E. coli* BL21(DE3) for protein expression.

Transformants expressing MBP-SUS1 proteins were induced with 0.5 mM IPTG at 30°C, cell pellets were washed and resuspended in 20 mM Tris pH 7.4, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, 10 µM leupeptin, 0.5 mM AEBSF, 1 mM benzamidine, 5 mM caproic acid and 1
µM E64. Cells were lysed by sonication in an ice bath, and clarified extracts were produced by two sequential centrifugations at 35,000g and 4°C for 15 min. Batch binding with amylose resin (New England Biolabs) equilibrated in column buffer (20 mM Tris pH 7.4, 200 mM NaCl, 1 mM DTT, 1 mM EDTA) was performed on ice for 20 min, washing with 15 bed volumes of column buffer and elution with 10 bed volumes of column buffer plus 20 mM maltose was performed in gravity-feed columns at 4°C. Dialyzed samples were loaded onto SOURCE 15Q (Amersham, Piscataway, NJ, USA) anion-exchange resin in Buffer A (50 mM MOPS pH 7.5, 2 mM DTT) and eluted at 4°C with a 30-ml linear gradient of 0-500 mM NaCl in Buffer A. Peak fractions were dialyzed against 10 mM MOPS pH 7.5, 1 mM DTT at 4°C.

Transformants expressing 6His-SUS1 proteins were induced with 0.5 mM IPTG at 30°C, cell pellets were washed and resuspended in 50 mM MOPS pH 7.5, 300 mM NaCl, 50 mM sucrose, 250 µg ml⁻¹ lysozyme, and incubated at room temperature for 30 min. Prior to sonication in an ice bath, 10 µM leupeptin, 1 mM AEBSF, 1 mM benzamidine, 5 mM caproic acid, 1 µM E64 and 0.2 M sucrose were added. Clarified extracts were produced by two sequential centrifugations at 35,000g and 4°C for 15 min. Batch binding with Talon metal affinity resin (Clontech, Palo Alto, CA, USA) equilibrated in column buffer (50 mM MOPS pH 7.5, 300 mM NaCl, 50 mM sucrose) was performed at 4°C for 30 min, washing with 26 bed volumes of column buffer plus 2.5 mM imidazole and elution with 10 bed volumes of column buffer plus 250 mM imidazole was performed in gravity-feed columns at 4°C. Samples were immediately adjusted to 1 mM DTT, 5 mM EDTA. Dialyzed samples were loaded onto SOURCE 15Q (Amersham) anion-exchange resin in Buffer B (50 mM MOPS pH 7.5, 1 mM DTT, 50 mM sucrose) and eluted at 4°C with a 30-ml linear gradient of 0-500 mM NaCl in Buffer B. Peak fractions were dialyzed against 10 mM MOPS pH 7.5, 1 mM DTT, 10 mM sucrose at 4°C. Protein concentrations were determined in duplicate by the dye-binding assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. Concentration of protein samples was performed by centrifugal filtration at 4°C in 10K MWCO Centricons (Millipore, Bedford, MA, USA).

Size exclusion chromatography was performed by loading 200 µl of recombinant MBP-SUS1 samples onto a Superdex 200 HR 10/30 column (Amersham) equilibrated in 10 mM MOPS pH 7.5, 1 mM DTT, 100 mM NaCl. Isocratic elutions were at 4°C and a flow rate of 0.5
ml min⁻¹ and 0.25 ml fractions were collected. The column was calibrated with thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa) and carbonic anhydrase (29 kDa).

**Ultracentrifugal Fractionation and Sucrose Gradient Flotation of Membranes**

Bacterial protein extracts from MBP-SUS1 transformants (in 50 mM MOPS pH 7.5, 1 mM DTT, 1 mM EDTA, 50 mM NaCl, 10 µM leupeptin, 1 mM AEBSF, 0.5 mM benzamidine, 1 µM E64, 0.25 M sucrose) and 6His-SUS1 transformants (in 50 mM MOPS pH 7.5, 1 mM DTT, 1 mM EDTA, 300 mM NaCl, 10 µM leupeptin, 1 mM AEBSF, 1 mM benzamidine, 5 mM caproic acid, 1 µM E64, 0.25 M sucrose) were clarified of any intact cells and inclusion bodies by filtration through Miracloth (Calbiochem) and two sequential centrifugations at 9,000-10,000g and 4°C for 30 min. Membrane vesicles within these extracts were recovered at 100,000g for 1.5-2.5 h at 4°C and the supernatants retained as the soluble phase extract (100K supers). Membrane vesicles were washed in 50 mM Mops pH 7.5, 1 mM DTT, 1 mM EDTA, 10 µM leupeptin, 1 mM AEBSF, 0.5 mM benzamidine, 1 µM E64, 0.25M sucrose, 150 mM NaCl and 0.05 % (w/v) Brij 58. The membrane fraction was pelleted at 100,000g for 1.5 h at 4°C and resuspended on ice in 10 % of the original volume in a 60 % (w/w) sucrose solution made in 50 mM Mops pH 7.5, 1 mM DTT, 0.1 mM EDTA, 0.5 mM benzamidine, 2.5 mM caproic acid, 1 µM E64. This sample was used as the pelleted fraction (100K pellets) and for flotation as previously described (Ahola et al., 1999; Hardin et al., 2004).

**Microsome Binding Experiments**

The ability of various SUS1 constructs to bind plant leaf microsomes was determined by pelleting or adapting the sucrose gradient flotation conditions to a microscale format. For the flotation assays, equimolar amounts of each recombinant SUS1 protein (10 or 15 pmol) was mixed with microsomes in 50 mM MOPS pH 7.5 or 50 mM MES pH 5.5, 150 mM sucrose on ice for 30 min. Membranes were pelleted in an airfuge (Beckman Coulter, Palo Alto, CA, USA) at 100,000g for 15 min and a portion of the supernatant (input) removed. The pellets were resuspended in 50 µl of a 60 % (w/w) solution of sucrose made in 50 mM MOPS pH 7.5 or 50 mM MES pH 5.5, and then overlaid with 175 µl of 50 % (w/w) sucrose and 15 µl of 10 % (w/w) sucrose both made in 50 mM MOPS pH 7.5 or 50 mM MES pH 5.5, 100 mM NaCl. The gradients were centrifuged at 100,000g for 1 h and the top 17-30 % of the gradient sampled.
Some samples were delipidated using a methanol-chloroform-water procedure (Wessel and Flugge, 1984) prior to SDS-PAGE analysis. For the pelleting assays, recombinant SUS1 proteins were ensured to be soluble by pre-spinning at 145,000g for 15 min in an Airfuge, and on occasion the tubes used for the binding reactions were blocked with a 0.1 % (w/v) solution of purified casein (I-block, Tropix, Bedford, MA, USA) in 50 mM MOPS pH 7.5. An equimolar amount of each recombinant SUS1 protein (11-22 pmol) was mixed with microsomes in 50 mM MOPS pH 7.5, 250 mM sucrose or 50 mM MES pH 6.5, 150 mM sucrose, glucose or maltose on ice for 30 min. Membranes were pelleted in an airfuge (Beckman Coulter, Palo Alto, CA, USA) at 145,000g for 15 min and a portion of the supernatant (input) removed. The pellets and tubes were washed with the appropriate buffered, sugar solution and then denatured for SDS-PAGE analysis.

**Enzyme Activity Assays**

SUS activity was assayed in the catabolic (cleavage) direction as described (Hardin et al., 2004) using a fixed-time assay in 50 mM MES pH 6.5 containing 1 mM UDP and 100 mM sucrose. Production of UDP-glucose was determined enzymatically using UDP-glucose-dehydrogenase (Calbiochem) coupled to the reduction of NAD.

**Immunological Methods**

The SUS1 peptides PH (CHILRVPFRTENGIVRKWISR) and NT (MGEGAGDRVLSRL) were purified by HPLC, verified by MS and used to generate immune serum in rabbits (Bethyl Laboratories, Montgomery, TX, USA). Immunosorbent columns coupled with PH or NT peptides were used to obtain the affinity-purified anti-SUS-PH and anti-SUS-NT antibodies. The anti-MBP polyclonal antiserum was from a commercial vendor (New England Biolabs). Proteins were denatured, separated on 7%-10% polyacrylamide-0.1% SDS gels, transferred onto polyvinylidene fluoride membranes (Immobilon-FL, Millipore, Bedford, MA, USA) and immunoblotted. Blocking was performed in 2 % (w/v) fish gelatin (Sigma) in PBS (5 mM NaH$_2$PO$_4$ pH 7.4, 150 mM NaCl), washes and antibody dilutions were done in PBS containing 0.1 % (v/v) Tween-20 (PBST). The Alexa Fluor 680-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) were detected by scanning on an Odyssey infrared imager (LI-COR, Lincoln, NE, USA) and densitometry performed with the supplied software.

**ACKNOWLEDGMENTS**
Mention of a trademark or proprietary product does not constitute a guarantee or warranty by the U.S. Department of Agriculture (USDA)-Agricultural Research Service and does not imply its approval to the exclusion of other products that might also be suitable.

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Konishi T, Ohmiya Y, Hayashi T (2004) Evidence that sucrose loaded into the phloem of a poplar leaf is used directly by sucrose synthase associated with various β-glucan synthases in the stem. Plant Physiol 134: 1146-1152


FIGURE LEGENDS

Figure 1. SUS proteins can be modeled onto a GT-B type glucosyltransferase fold and contain a region with sequence similarity to a PH-domain. A, Alignment of amino acid sequences of the maize SUS isoforms: SUS1 (P49036), SUS-SH1 (P04712) and SUS2 (Q8L5H0) with the C-terminal PH-domain of human pleckstrin (HsPlek; P08567) by CLUSTAL W (v 1.82). Secondary structure elements for SUS1 identified by the Jpred method are shown above the sequences, and those of pleckstrin below the sequences. Conservation of similar amino acids between HsPlek and the SUS isoforms is indicated by gray shading. Consensus residues are conserved across all four proteins: h, hydrophobic; a, acidic; b, basic residues. The locations of Ala-substituted mutants are indicated by "A" and truncation (stop) mutants with arrows above the SUS1 sequence, residues identified in the present study as important for membrane/lipid binding are marked with asterisks. Asterisks below the HsPlek sequence are important for PH-domain membrane binding (Isakoff et al., 1998; Edlich et al., 2005). B, Diagram of SUS1 sequence showing Jpred secondary structural elements (α-helices as circles, β-strands as arrows) for the putative catalytic domain on a GT-B type fold (drawn after MacGregor, 2002).
lines represent the PH-like domain of SUS1, locations of truncation mutants are indicated by "stop", and Ala-substituted mutants by an "A". The putative catalytic glutamate residues are shown in gray.

**Figure 2.** SUS1 truncation mutants partition to the membrane-containing insoluble phase in *E. coli*. Equal amounts of total protein (0.25 µg) isolated in *E. coli* 100,000g supernatants (100K supers) and pellets (100K pellets) of recombinants expressing full length SUS1 or the truncation mutant listed above the panels were probed on immunoblots (IB) with an antibody against MBP (anti-MBP). The positions of PAGE molecular mass markers are shown in kilodaltons (kDa) on the left of each panel. Densitometry of these blots is displayed in the graph at the bottom.

**Figure 3.** Size exclusion chromatography demonstrates that SUS1 truncation mutants are all dimers. Equal volumes of total protein isolated in *E. coli* 100,000g supernatants of recombinants expressing full length SUS1 or the truncation mutant listed to the left of the panels were resolved by size-exclusion chromatography (SEC) and fractions probed on immunoblots (IB) with an antibody against MBP. The elution positions of SEC molecular mass markers are shown above the panels, the calculated monomer molecular weight (MW) of each protein and its deduced oligomeric type are listed to the left of the panels.

**Figure 4.** Sucrose gradient flotation reveals that the N-terminal 362 amino acids of SUS1 binds to bacterial membranes. Equal volumes of total protein isolated in *E. coli* 100,000g pellets of recombinants expressing full length SUS1 or the truncation mutant listed to the left of the panels were subjected to sucrose gradient flotation and fractions probed on immunoblots (IB) with an antibody against MBP. Densitometry of these blots is displayed in the graph at the bottom.

**Figure 5.** Sucrose gradient flotation reveals that the N-terminal 362 amino acids of SUS1 binds to plant membranes. Equal amounts (0.18 pmol µl\(^{-1}\)) of full length SUS1 or the truncation mutant listed above the panels were mixed with equal amounts of basal leaf microsomes at pH 5.5 and 150 mM sucrose. After pelleting the microsomes and removing aliquots of the supernatants (input) the pellets were subjected to sucrose gradient flotation and the top fraction probed on immunoblots (IB) with an antibody against MBP. Densitometry of these blots is displayed in the graph at the bottom. Note that free MBP did not bind to membranes.

**Figure 6.** The source region of leaf microsomes affects membrane binding of SUS1 implicating multiple membrane-interacting protein regions. Equal amounts (0.28 pmol µl\(^{-1}\)) of MBP, full length MBP-SUS1 or the T362\(_{stop}\) truncation mutant were mixed with equal amounts of basal or...
apical leaf microsomes (before or after being carbonate stripped) at pH 7.5 and 250 mM sucrose. The microsomes were pelleted, aliquots of the supernatants removed before washing the pellets and then both were probed on immunoblots (IB) with an antibody against MBP.

**Figure 7.** Sucrose gradient flotation reveals that the PH-like domain of SUS1 influences binding to bacterial membranes. A, Equal volumes of total protein isolated in *E. coli* 100,000g pellets of recombinants expressing wild type SUS1 or the Ala substituted mutant listed to the left of the panels were subjected to sucrose gradient flotation and fractions probed on immunoblots (IB) with an antibody against the SUS1 N-terminus (anti-SUS-NT). Densitometry of these blots is displayed in the graph at the bottom. B, Sucrose cleavage activity (µmol UDP-Glc min⁻¹ mg⁻¹) of wild type SUS1 and the Ala substituted mutants. Y-axis bars indicate standard errors.

**Figure 8.** Sucrose gradient flotation reveals that the PH-like domain of SUS1 influences binding to plant microsomes. Equal amounts (0.30 pmol µl⁻¹) of wild type SUS1 or the Ala substituted mutants listed above the panels were mixed with equal amounts of apical leaf microsomes at pH 7.5 and 150 mM sucrose. After pelleting the microsomes and removing aliquots of the supernatants (input) the pellets were subjected to sucrose gradient flotation and the top fraction probed on immunoblots (IB) with an antibody against SUS1 (anti-SUS-PH). A representative immunoblot is shown and densitometry of three separate blots is displayed in the graph at the bottom. Y-axis bars indicate standard errors.

**Figure 9.** Sugars stimulate binding of SUS1 to plant microsomes. Equal amounts (0.75 pmol µl⁻¹) of full length MBP-SUS1 were analyzed without added microsomes or mixed with equal amounts of carbonate-stripped apical leaf microsomes at pH 6.5 and residual (4 mM) sucrose (duplicate reactions shown) or after addition of 150 mM sucrose (duplicate reactions shown), glucose or maltose. The controls and microsomes were pelleted, washed and then probed on immunoblots (IB) with an antibody against MBP. Densitometry of these blots is displayed in the graph at the bottom.

**Figure 10.** Low pH and high SUS1 protein concentration positively influence binding to plant microsomes. A, Equal amounts (0.18 pmol µl⁻¹) of full length MBP-SUS1 were mixed with equal amounts of apical leaf microsomes at pH 5.5 or pH 7.5 and 150 mM sucrose. After pelleting the microsomes and removing aliquots of the supernatants (input) the pellets were subjected to sucrose gradient flotation and the top fraction probed on immunoblots (IB) with an antibody against MBP. Densitometry of these blots is displayed in the graph at the bottom, Y-
axis bars indicate standard errors. B, Increasing amounts (0.023-0.36 pmol µl⁻¹) of full length MBP-SUS1 were mixed with equal amounts of carbonate-stripped apical leaf microsomes at pH 5.5 and 150 mM sucrose. After pelleting the microsomes and removing aliquots of the supernatants (input) the pellets were subjected to sucrose gradient flotation and the top fraction probed on immunoblots (IB) with an antibody against MBP. Densitometry of these blots is displayed in the graph at the bottom.
A

2° structure

**Structure Diagram**

Ala-mutants
SUS1 (360) LGTEHCHILRVPFTENGILRQFSERFEWYPTEFTSVEIAGKMQAPD
SUS-SH1 (352) LGTEHTDIIRVPFRNEGILRQFSERTADWPEYDVSSEIKMEOAKPD
SUS2 (355) SGTQHTLYLVPFRNEGILRQFSDWPEYDVTAFEADAEGIAKLGTD
HsPlek (244) VIIVQGCLLQGHKRWKRKLQKFLRED-PAYLYHDGPAGAEDPLGAIHLRGCC

Consensus

2° structure

**Consensus Sequence**

SUS1          LGTEHCHILRVPFTENGILRQFSERFEWYPTEFTSVEIAGKMQAPD (360)
SUS-SH1       LGTEHTDIIRVPFRNEGILRQFSERTADWPEYDVSSEIKMEOAKPD (352)
SUS2          SGTQHTLYLVPFRNEGILRQFSDWPEYDVTAFEADAEGIAKLGTD (355)
HsPlek        VIIVQGCLLQGHKRWKRKLQKFLRED-PAYLYHDGPAGAEDPLGAIHLRGCC (244)

Consensus

2° structure

**Consensus Structure**

B

**Figure 1**

SUS1 N-terminal (280 AA) non-catalytic domain

SUS1 C-terminal (47 AA) non-catalytic domain

Linker

**Diagram of SUS1 Structure**

α8

α7

α6

α5

α4

α3

β

β

β

β

β

β

β

β

β

Figure 1
Figure 2

MBP-SUS1 soluble (100K supers)

IB: anti-MBP

MBP-SUS1 insoluble (100K pellets)

IB: anti-MBP

Bacterial intracellular distribution

% of total in pellet

T362 stop  Q408 stop  S465 stop  Y511 stop  MBP-SUS1

Figure 2
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**Figure 3**
Figure 4
Figure 5
Figure 6

MBP-SUS1 pelleted with plant microsomes

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IB: anti-MBP

[Image of gel electrophoresis results]

kDa: 113, 91, 50, 35
Figure 7

(A) 6His-SUS1 bacterial membrane binding

(B) 6His-SUS1 sucrose cleavage activity
Figure 8

6His-SUS1 plant microsome binding

IB: anti-SUS-PH

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Figure 9
Figure 10