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A sucrose binding protein homologue from soybean exhibits GTP-binding activity that functions independently of sucrose transport activity

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The sucrose binding protein (SBP) has been implicated as an important component of the sucrose uptake system in plants. SBP-mediated sucrose transport displays unique kinetic features and the protein is not similar to other transport proteins. Here, we report the characterization of a member of the SBP family from soybean [*Glycine max* (L) Merrill] designated S64 or SBP2. Subcellular fractionation and precipitation by GTP-agarose demonstrated that S64/SBP2 is a membrane-associated protein that exhibits GTP binding activity. Purified recombinant S64/SBP2 protein, expressed as a histidine-tagged protein in *Escherichia coli*, exhibited nucleotide-binding specificity to guanine nucleotides. The GTP binding site was mapped to an imperfect Walker A type-sequence, Ala279-Leu-Ala-Pro-Thr-Lys-Lys-Ser286, by site-directed mutagenesis. *Escherichia coli*-produced wild-type protein and a truncated version of the protein containing the putative binding-sequence-bound GTP, although not with the same efficiency. In contrast, replacement of Thr283 and Lys284 residues to Leu and Glu residues prevented GTP binding. The site directed mutant failed to bind GTP but retained the ability to undergo oligomerization and to promote growth of the susy7 yeast strain, deficient in utilizing extracellular sucrose, on medium containing sucrose as the sole carbon source. Our results indicate that GTP binding and sucrose transport by SBP are separable and function independently. The implications of our findings with respect to the function and membrane topology of SBP are discussed.

Keywords: sucrose transporter; soybean; yeast complementation assay; *Glycine max*.

In many higher plants, sucrose is the predominant form of photoassimilate that is transported from mature leaves (source tissues) to sink tissues, such as seeds, stems, reproductive organs and roots, via the vascular system [1]. Biochemical studies have demonstrated that sucrose uptake kinetics in leaves is complex and consists of multiple components; for example, in *Vicia faba*, two saturable (high- and low-affinity) components and one linear, low-affinity component have been described [2]. Our understanding of sucrose translocation has advanced considerably over the last decade with the molecular and biochemical characterization of the sucrose transporter (SUT) family of low- and high-affinity sucrose transporters [1]. The SUT1 protein has been described as the proton-motive-force-driven sucrose symporter that mediates phloem loading and long-distance transport, the key transport step in assimilate partitioning for many plants [3–5]. SUT1 serves as a high-affinity transporter, whereas SUT4, a second member of this sucrose transporter family, corresponds to the low-affinity/high capacity saturable component of sucrose uptake found in leaves [6]. A third structurally related-member of the family has been identified and designated SUT2 [7]. The SUT2 protein has been proposed to act as a sugar sensor that controls sucrose fluxes across the plasma membrane of sieve elements by regulating expression, activity and turnover of SUT1 and SUT4 [7]. This hypothesis was raised based on the lack of transport activity of SUT2 and its colocalization with the high and low-affinity sucrose transporter in sieve elements. Nevertheless, direct evidence for a SUT2 sucrose sensor and regulatory function has not been provided.

Earlier attempts to identify sucrose transporters resulted in the identification of a sucrose binding protein from soybean cotyledonary microsomal membrane fraction by its capacity to bind to the sucrose analogue 6′-deoxy-6′-(4-azido-2-hydroxy)-benzamido-sucrose [8]. Subsequent progress in characterizing SBP led to the isolation of its cDNA from an expression library prepared from cotyledon mRNA [9]. Molecular characterization of the cDNA-encoded product revealed that SBP was quite dissimilar from the H^+/sucrose symporter SUT. Despite the lack of similarity between SBP and other known membrane transport proteins, several lines of evidence have implicated the SBP protein as the linear, low affinity component of sucrose uptake system in plants. The SPB protein is localized in the plasma membrane of cells that are actively engaged in sucrose transport, such as mesophyll cells of

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Abbreviations: SUT, sucrose transporter; SBP, sucrose binding protein; CaMV, cauliflower mosaic virus; rbcS, small subunit of RUBISCO; ADH, alcohol dehydrogenase; DAF, days after flowering.

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young sink leaves, the companion cells of mature phloem and the cells of cotyledons undergoing differentiation [9,10]. In the cotyledon, expression of the *SBP* gene is temporally regulated and accumulation of the protein is coordinated with active sucrose uptake [9]. In spinach, a SBP homologue was immunolocalized in the plasma membrane of sieve elements in fully expanded leaves, shoots and roots [11,12] and in *V. faba* developing seeds, SBP was colocalized with the H^+/sucrose symporter in the plasma membrane of transfer cells [13]. A SBP homologue was also detected in the microsomal fraction of young leaves from *Nicotiana tabacum* [14]. Direct evidence implicating SBP in sucrose transport has been obtained with complementation studies using a secreted-invertase-deficient mutant yeast strain, incapable of growth on medium containing sucrose as the only carbon source [15,16]. Ectopic expression of the *SBP* cDNA alone reverses the mutant yeast phenotype and SBP-mediated specific sucrose uptake in yeast displays linear, nonsaturable kinetics up to 30 mM external sucrose, being relatively insensitive to pH gradient across the membrane [15,17]. These biochemical features closely resemble the kinetics properties of the previously characterized linear component of sucrose uptake in higher plants [18–20]. Recently, we have conducted overexpression and antisense repression studies in transgenic tobacco (*Nicotiana tabacum* L. Cv Havana) to analyze the function of SBP in the long-distance sucrose transport [14]. The antisense transgenic plants developed symptoms consistent with inhibition of sucrose translocation and displayed a reduction in plant growth and development. Furthermore, both antisense repression and overexpression of a SBP homologue in transgenic lines altered carbohydrate partitioning in mature leaves. These results indicated that SBP might represent an important component of the sucrose translocation pathway in plants.

More recently, we have addressed the role of SBP in plant cell sucrose transport by performing radiolabeled sucrose uptake experiments with transgenic tobacco cell lines expressing the *SBP* sense or antisense gene [21]. In this condition, the level of a SBP homologue correlated with the efficiency of radiolabeled uptake by the transgenic tobacco cells. Furthermore, manipulation of SBP levels altered sucrose-cleaving activities in a metabolic compensatory manner. Enhanced accumulation of SBP caused an increase in intracellular sucrose synthase activity with a concomitant decline in cell-wall invertase activity. This alteration in sucrose-cleaving activities is consistent with a metabolic adjustment of the sense cell lines caused by its high efficiency of direct sucrose uptake as disaccharide. Although these studies clearly demonstrated that SBP is involved in sucrose translocation-dependent physiological processes, still unresolved is whether the underlying mechanism involves SBP-mediated sucrose transport or SBP-mediated regulation of alternative carbohydrate uptake systems.

Despite the functional characterization of SBP, potential post-translational modifications that could regulate its function have not been examined. In this investigation, we describe the identification of an isoform of soybean SBP, designated S64 or SBP2, and we show that the SBP homologue is a membrane-associated GTP binding protein. We have generated mutants that blocked its GTP binding activity but not interfered in its oligomerization property and S64/SBP-mediated sucrose transport in yeast. These mutants should be valuable tools for determining the physiological role of SBP as a G-protein *in vivo*.

EXPERIMENTAL PROCEDURES

Isolation of a SBP homologue cDNA from soybean

DNA manipulations were performed essentially as described previously [22]. The *S64* cDNA (GeneBank accession number AF191299) was unintentionally isolated from a soybean seed expression library using an antibody raised against a partially purified microsomal membrane fraction from immature soybean seeds [14]. The positive clones resulted from this screening were designated by the letter S from soybean seeds followed by 1 : 1000 of the estimated M_r of the encoded product. The identity of this particular *S64* clone was obtained by sequence comparison analysis using the BLAST program [23]. The computer program CLUSTALW was used for sequence alignment. The S64 deduced protein shares 86% sequence identity with the sucrose binding protein (GeneBank accession number L06038) and is also referred to as SBP homologue or SBP2.

Construction of plasmids and antibody production

The *S64*/*SBP* homologue insert was released from the λ recombinant DNA with *Eco*RI digestion and subcloned into the *Eco*RI site of pUC118 to obtain the clone pUFVS64. The S64 protein was expressed as a fusion protein using the pET-16b vector (Novagen), which provides an N-terminal His tag. For this purpose, an *Eco*RI site immediately adjacent to the stop codon was created by PCR using the *Pfu* DNA polymerase, the forward primer S64XHOF 5′-AAGAAA*C*TCGAGGTCGAAGA-3′ (coordinates 103–121, *Xho*I site underlined) and the reverse primer SEF97R 5′-ATACATTCCCCGAATTCAGCCA CCTCC-3′ (positions 1498–1524, *Eco*RI site underlined). The amplified sequence, spanning the entire protein-coding region and lacking the putative peptide signal coding sequence and the 3′ untranslated sequences, was subcloned into the *Eco*RI/*Sma*I-restricted pGEM7Zf(–) vector (Promega), and then moved as a *Xho*I insert into pET16b, yielding pUFV120.

The construction was transformed into *E. coli* strain BL21 (DE3) and the synthesis of the recombinant protein was induced by isopropyl thio-β-D-galactoside (IPTG). The induced protein was affinity-purified using Ni-chelating Sepharose resin (Amersham Pharmacia Biotech.) and used as an antigen to raise polyclonal antisera in rabbits, which were immunized through subcutaneous injections during 2-week intervals. The specificity of the anti-S64 serum was previously evaluated with protein extracts from transgenic tobacco plants expressing the *S64*/*SBP* homologue cDNA either in the sense or antisense orientation [14,21], in a yeast expression system [14] and in a bacteria expression system [14].

Truncated protein, mutagenesis and bacterial overexpression

To produce a S64 truncated protein, an internal 916-bp sequence of *S64* cDNA was released from pUFVS64 with

*Sau*3AI digestion and inserted into the *Bam*HI site of pET16b to create pUFV50. The inserted sequence spans nucleotides 122–1041 of the cDNA and encodes the amino acid residues from position 36–343.

The putative GTP-binding site was mutated using a PCR-based mutagenesis strategy, in which overlapping upstream and downstream sequences of the site were individually amplified with sets of primers to create an internal *Xba*I site within the putative site. The sets of primers used were SPI97F 5′-TCCTCACTGCAG TCACCATGGCGACCA-3′ (coordinates 1–27, *Pst*I site underlined) and SGTPXBAF 5′- GGCCCCTCTAGA GAAAAGCTC-3′ (coordinates 857–878, *Xba*I site underlined) for the S64 N-terminal encoding sequence as well as SGTPXBAR 5′-GCTTTTCTCTAGAGGGGCC AACG-3′ (positions 853–876, *Xba*I site underlined) and SEF97R 5′-ATACATTCCCCGAATTCAGCCACC TCC-3′ (positions 1498–1524, *Eco*RI site underlined) for the adjacent C-terminal encoding sequence. The upstream-amplified sequence was digested with *Pst*I and *Xba*I, whereas the downstream-amplified fragment was digested with *Xba*I and *Eco*RI, and then they were inserted by triple ligation into *Pst*I–*Eco*RI sites of pUC118 to obtain pUFV193. This restored the *S64* coding region in which an internal *Xba*I site was created and, as consequence, the putative GTP-binding site Ala279-Leu-Ala-Pro-Thr-Lys-Lys-Ser286 was mutated to Ala279-Leu-Ala-Pro-Leu-Glu-Lys-Ser286. The mutations were confirmed by sequencing. To transfer the mutated *S64* sequence to pET16b, it was amplified from pUFV193 with the sense primer S64XHOF and the antisense primer SEF97R. The amplified sequence, harboring the mutated protein-coding region and lacking the putative peptide signal coding sequence and the 3′ untranslated sequences, was subcloned into the *Eco*RI/*Sma*I-restricted pGEM7Zf(–) vector (Promega), and then moved as a *Xho*I insert into pET16b to obtain pUFV232.

Constructions in pET16b were expressed in *E. coli* strain BL21 (DE3) LysS following induction by IPTG. N-Terminal His-tagged SBP fusion proteins were purified according to manufacturer's instructions (Novagen) for soluble proteins. For oligomerization studies, after a first round of purification, the His tag was removed from the *E. coli*-produced proteins by treatment with catalytic amounts of Factor Xa (10 $\mu g \cdot mg^{-1}$ of recombinant protein) in 100 mM NaCl, 50 mM Tris/HCl, pH 8.0, 1 mM $CaCl_2$ at 37 °C for 24 h.

Isolation of microsomal fraction

For microsomal membrane isolation, soybean cotyledons were homogenized with 25 mM Tris/HCl, pH 7.0, 250 mM sucrose, 2.5 mM dithiothreitol, 10 mM $MgSO_4$, 0.5% (w/v) gelatin and 0.5 mM phenylmethanesulfonyl fluoride [8]. The homogenate was filtered and centrifuged for 15 min at 13 000 ***g*** and 4 °C. Microsomal preparations were isolated by centrifugation at 80 000 ***g*** for 45 min [24].

Transient expression of S64/SBP homologue in soybean suspension cells

The pUFVS64 clone was modified by site-directed mutagenesis to create an *Eco*RI restriction site immediately downstream of the stop codon, yielding pUFV32. A plant expression cassette containing the *S64*/*SBP* homologue gene was constructed by insertion of the *S64* coding region that was released from pUFV32 with *Eco*RI/*Bam*HI digestion into pMON921 vector [25], previously digested with *Bgl*II/*Eco*RI. The resulting plasmid, pUFV52, harbors the *S64* coding region in the sense orientation placed between the *CaMV 35S* promoter with a duplicated enhancer region and the 3′ end of the pea *E9 rbcS* gene. A soybean cell culture line was generated and established as described previously [26]. Transient expression of *S64* was performed by electroporation (380 V, 975 μF) of 10 μg of expression cassette DNA and 40 μg of sheared salmon sperm DNA into 0.8 mL of cultured soybean cells in electroporation buffer (80 mM KCl, 5 mM $CaCl_2$, 10 mM Mes, pH 6.7, 0.425 M mannitol). Prior to electroporation, soybean suspension cells at 4 days after passage were recovered by centrifugation at 200 ***g***, washed three times and concentrated twice with electroporation buffer, incubated with plasmid and carrier DNA at 37 °C for 1 h and then at 0 °C for 10 min. The electroporated cells were diluted into 10 mL of MS medium [27], supplemented with complex vitamin B5, 0.2 $mg \cdot mL^{-1}$ 2,4-dichlorophenoxyacetic acid, 6% (w/v) sucrose and 15 mM glutamine, pH 5.7. Total protein was isolated from cells 48 h after transfection as described [24], separated by SDS/PAGE and immunoblotted with anti-S64 serum.

Gel electrophoresis and immunoblotting analysis

SDS/PAGE was carried out as described previously [28] and the proteins were transferred from 10% SDS/polyacrylamide gels to nitrocellulose membrane by electroblotting. The membrane was blocked with 3% (w/v) BSA in NaCl/Tris/Tween [100 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween-20]. S64/SBP homologue was detected using polyclonal anti-S64 serum at a 1 : 1000 dilution, followed by a goat anti-(rabbit IgG) Ig conjugated to alkaline phosphatase (Sigma) at a 1 : 5000 dilution. Alkaline phosphatase activity was assayed using 5-bromo-4-chloro-3-indolyl phosphate (Life Technologies, Inc.) and *p*-nitroblue tetrazolium (Life Technologies, Inc.).

Binding of S64/SBP homologue to GTP-agarose

Whole cell protein extracts were obtained from transgenic tobacco cell lines expressing a soybean *S64* transgene [21] and from soybean suspension cells transiently transformed with a *S64* expression cassette. Protein extracts were prepared by homogenization of the cells with lysis buffer [100 mM Tris/HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 0.1 mM dithiothreitol, 5 mM $MgCl_2$] at a ratio of 1 mg of cells per 2 μL of buffer and then clarified by centrifugation at 20 000 ***g*** for 20 min. The supernatant (2 mL) was incubated with 100 μL of 50% (v/v) GTP-agarose suspension in 50 mM Tris/HCl, pH 7.5, for 12 h under agitation at 4 °C [29]. The agarose beads were pelleted by centrifugation, washed extensively with cold 50 mM Tris/HCl, pH 7.5 and resuspended in 40 μL of SDS/PAGE sample buffer.

GTP bound proteins were fractionated by SDS/PAGE, transferred to nitrocellulose and probed with anti-S64 serum, as described above.

Binding of S64/SBP homologue fusion protein to nucleotide-agarose

The purified His-tagged S64 fusion protein (2 μg) was incubated with either 50 μL of ATP-agarose, GTP-agarose or Protein A-agarose suspension, previously equilibrated with binding buffer [20 mM Tris/HCl pH 7.5, 100 mM NaCl, 0.01% (v/v) Triton X-100, 2 mM $MgCl_2$]. After 1 h at 4 °C, the beads were washed three times with 500 mL of cold binding buffer and eluted in SDS/PAGE sample buffer at 100 °C for 3 min. The eluted proteins were separated by SDS/PAGE and stained with Coomassie Blue. In competition assays, GTP, GDP, GTPγS (guanosine 5′-*O*-3-thiotriphosphate), ATP, UTP or CTP were included in the binding buffer at 2 mM and incubated with the recombinant protein (0.5 μg) for 30 min at 4 °C prior to the binding reaction to GTP-agarose.

GTP-binding assay

The GTP-binding assay was performed as described previously [30]. Briefly, *E. coli*-expressed His fusion proteins were affinity-purified and blotted onto nitrocellulose membrane using BIO-DOT™ (Bio-Rad), according to the manufacture's instructions. Alternatively, affinity-purified recombinant proteins were fractionated by SDS/PAGE and transferred to nitrocellulose by electroblotting. The membranes were washed twice with binding buffer [50 mM NaH_2PO_4, pH 7.5, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.3% (v/v) Tween-20 and 4 μM ATP] and then incubated with binding buffer supplemented with 1 $\mu C \cdot mL^{-1}$ (or 0.33 nM) [α-^{32}P]GTP (3000 $Ci \cdot mmol^{-1}$; Amersham/Pharmacia) for 2 h. After the incubation period, the membranes were washed at least six times with binding buffer and subjected to autoradiography at −80 °C, using WOLF L-PLUS 505504 LP intensifying screens (Sigma).

Yeast strain and plasmids

The generation of *susy*7 yeast strain has been described previously [3]. It has the potato sucrose synthase gene stably integrated into its genome but lacks an endogenous sucrose transport system and invertase activity. Thus, *susy*7 yeast strain is incapable to grow on a medium containing sucrose as the sole carbon source, unless a sucrose uptake system is provided through ectopic expression. For complementation assays in the mutant yeast strain, the intact *S64* cDNA was released from pUFVS64 with *Eco*RI and inserted into the same site of the yeast expression vector 112AINE [3]. The resulting plasmid, pUFV373, contains the *S64* cDNA in the right orientation placed between the *ADH1* promoter and 3′ end of *ADH1* gene. A yeast expression cassette containing the mutated *S64* gene was constructed by insertion of the GTP binding site mutated cDNA that was released from pUFV193 with *Pst*I and *Eco*RI digestion into the same restriction sites of the 112AINE vector. The resulting plasmid, pUFV375, harbors the mutated *S64* coding region in the sense orientation placed between the *ADH*1 promoter and the 3′ end of the *ADH*1 gene.

The *susy*7 yeast strain was transformed with either pUFV373 or pUFV375 by electroporation [31], resulting in *susy*7-S64 or *susy*7-MS64, respectively. To monitor growth on sucrose medium, 200 μL of a 24-h-old liquid culture of either *susy*7-S64 or *susy*7-MS64 growing in complete medium supplemented with 2% (w/v) glucose were used to inoculate 20 mL of complete medium with 2% (w/v) sucrose as the only carbon source. Relative growth was monitored by taking the D_{600} during 24-h intervals, as indicated in the figure legend. For each DNA construct, at least three independent transformants were monitored.

RESULTS

Isolation of a second member of the *SBP* gene family from soybean

Based on structural homology and functional analogy, we have isolated a sucrose binding protein (SBP) homologue cDNA from soybean. The predicted encoded protein was first designated S64, has an estimated M_r of 55 834 and pI of 6.32. Sequence comparison analysis revealed that the predicted encoded protein was quite similar to the sucrose binding protein, first identified in soybean cotyledon (86% sequence identity). It also showed a significant amino-acid sequence similarity to heterologous SBP sequences from other plant species (Fig. 1). Analysis of the deduced amino-acid sequence allowed us to predict a signal peptide and its processing site, which suggests that the protein be targeted to the secretory pathway. In fact, the S64 protein was detected in microsomal fraction of soybean cotyledon (Fig. 2).

In soybean cotyledon, the S64 antibody recognized two cross-reacting polypeptides with slightly different electrophoretic mobility (Fig. 2B, lane ME). Because SBP has a predicted M_r of 60 522 and it is highly homologous to S64, the reduced SDS/PAGE mobility polypeptide could represent SBP. Alternatively, the cross-reacting polypeptides could be differentially processed forms of the same S64/SBP homologue protein. The primary structure of the S64/SBP homologue protein shows the presence of a consensus sequence for nucleotide binding and a site for N-linked glycosylation, as potential sites for post-translational modifications of the protein (Fig. 1). Furthermore, despite the hydrophilic nature of SBP, solubilization and partitioning studies of plasma membrane proteins have demonstrated that ≈25% of SBPs are associated with a hydrophobic portion of the plasma membrane [10]. This observation has led to the suggestion that the putative leader peptide, which corresponds to the only hydrophobic region of the protein, is not quantitatively cleaved from the mature protein. The presence of the leader peptide in a fraction of S64/SBP homologue would explain the antibody cross-reactivity to the higher molecular mass form. To examine these possibilities, we transferred the *S64* coding region to a plant expression cassette and the recombinant protein was oversynthesized in cultured soybean cells (Fig. 3, compare lanes 1 and 2). In the homologous system, the apparatus for protein processing is expected to operate properly and with similar specificity. As shown in Fig. 3, the S64/SBP homologue protein was synthesized as single polypeptide (lane 1) that comigrated with the faster migrating polypeptide detected in membrane fraction of soybean cotyledon

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          1            15 16        ⇓  30 31           45 46           60 61           75 76           90
1 pUFVS64 MATRAKLSLAIFLFF LLALISNLALGKLKE TEVEE-DPELVTCKH QCQQQRQYTESDKRT CLQQCD---SMKQER EKQVEEETREKEEE-     85
2 SOYSBP  .GM.T......F.. ....F....F..C.. .....E......... .....Q....G...V ...S..RYHR..... ...IQ......K..E    90
3 p54     ..IKT....T..... ....LCSNLAVGR.. -----K....T.C.D ..DM....D.E...I .MER..DYIKK.... Q.HK.H.EE.EQ.Q-    84
4 VFSBP   ..IKT....T..... ....LCSNLAIAR.. -----K....T...D K.DL.G..D.E...I .MEK.EDYVRK.... Q.HK.H.KE.HE..-    84

          91          105 106         120 121         135 136         150 151         165 166         180
1 pUFVS64 ------HQEQHEEEQ DQNPYVFEEDKDFST RVETEGGSIRVLKKF TEKSKLLQGNENFRL AILEARAHTFVSPRH FDSEVVLFNIKGRAV    169
2 SOYSBP  SREREEE.QEQH... .E...I.......E. .......R....... .........I..... ..............  ......F........    180
3 p54     ---------EED..- ---.....-.N..E. KID.KD.RVLI.N.. N......KNI..YG. .V..IK.NA.L..H. Y...AI.......GI    159
4 VFSBP   ---------EEN..- ---.....-N...E. KID.KD.R.LI.N.. H......KNI..YG. .V..IK.NA.L..H. Y...AI......KGI    159
                                            L  F             R       P    P    D        G

          181         195 196         210 211         225 226         240 241         255 256         270
1 pUFVS64 LGLVRESETEKITLE PGDMIHIPAGTPLYI VNRDENEKLLLAMLH IPVST---PGKFEEF .GPGGRDPESVLSAF SWNVLQAALQTPKGK    256
2 SOYSBP  ....S.......... ..............  .......D.F..... ....VS-T....... .A.............  ..............    269
3 p54     I...A.DR..RFN.. E..IMRV.....M.L .........YI.AF. M.P.SGSA.VNL.P. .ESA..K.....NT. .SK......KSS..E    249
4 VFSBP   I...A.DQ..RFN.. E..L.RV.....T.L .........LI.AF. L.P.SGSA.VNL.P. .ES...R......T. .SK......KSSERE    249
                          G  Y           D                  E             F S

          271         285 286         300 301         315 316         330 331         345 346         360
1 pUFVS64 LERLFNQQNEGSIFK ISRERVRALAPTKKS SWWPFGGESKAQFNI FSKRPTFSNGYGRLT EVGPDD-EKSWLQRL NLMLTFTNITQRSMS    345
2 SOYSBP  ..NV.D........R ....Q.......... ..............  ......I........ ......D........ ..............    359
3 p54     ..TVLDE.KK.R... .EK.D..G...-... -L.....PF.SP... ..NN.A...KF.S.F ....SQ-...G.EG. .....LA...KG...    336
4 VFSBP   ..TVLDE.QK.R... .AK.D.LS...-.R- .L.....TF.GP... RNNN.A...QF.T.F ....SL-..TG.EG. ......A...KG...    336
            Q                                                N  G                          G

          361         375 376         390 391         405 406         420 421         435 436         450
1 pUFVS64 TIHYNSHATKIALVM DGRGHLQISCPHMSS R-SDSKHDKSSPSYH RISADLKPGMVFVVP PGHPFVTIASNKENL LIICFEVNVRDNKKF    434
2 SOYSBP  ..............I .............. ...H.......... ...S.......... .............. .M......A......    449
3 p54     .....TN.N.....I ..E.E.EMA....P. S..N.RQK...I... N.N.K.R..VM.... A.....N...K.K.. IVV.....AQR...L    426
4 VFSBP   .....TN.N.....V N.E.DFEMA....P. S..N..QK...I... N.N.K.......... A.....N...K.N.. LIL.....AQR...L    426
           I              G                                L       P        A              N

          451         465 466         480 481         495 496         510 511         525 526         540
1 pUFVS64 TFAGKDNIVSSLDNV AKELAFNYPSEMVNG VFERK---------- ----------ESLFF PFELP--SEERGRRA VA    489
2 SOYSBP  .............. .............. ..LLQRFLERKLIGR LYHLPHKDRK..F.. .....--R..... D.    524
3 p54     AL...K....A..KA ...V..DIAA.K.DE .....---------- ----------.EF.. .YDNEERK..H..AV .-    483
4 VFSBP   AF...K..M.A..KT ......DLAAQKVDK I....---------- ----------.E... .YD-EERK.....AF .-    482
             G              L F
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Fig. 1. Comparison of the amino acid sequence of S64 with SBP from soybean and other organisms. A multiple sequence alignment of the deduced amino acid sequence of soybean S64 (pUFVS64, GeneBank accession number AF191299), soybean SBP (SOYSBP, GeneBank accession number L06038), pea SBP homologue (p54, GeneBank accession number Y11207) and *Vicia faba* SBP homologue (VPSBP, GeneBank accession number VFA292221) was obtained with the CLUSTALW program. The amino acid sequences are in the one-letter code and have been aligned by introducing gaps (shown as dashes) to maximize identity. Dots represent identity to S64. The nucleotide-binding motif is boxed and the putative N-linked glycosylation site is underlined. The open arrow indicates the putative signal peptide cleavage site of S64. Amino acid residues indicated below the sequences correspond to highly conserved residues in equivalent positions of vicilin-like protein sequences that are important in maintaining their three-dimensional structure.

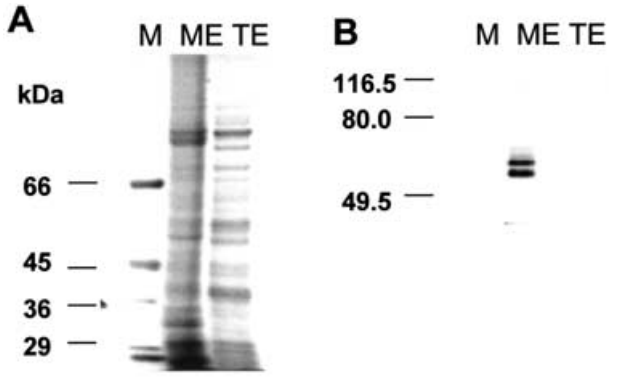

Fig. 2. SDS/PAGE and immunoblotting of membrane fractions from soybean cotyledons. (A) Whole cell protein extracts (TE) and microsomal membranes (ME) were isolated from soybean seeds at 20 days after flowering (DAF), fractionated by SDS/PAGE and stained with Coomassie Brilliant Blue. M corresponds to molecular mass markers indicated on the left in kDa. (B) SDS/PAGE fractionated protein was transferred to nitrocellulose membranes and probed with an anti-S64 serum.

(lane 3). This result indicates that the reduced SDS/PAGE mobility polypeptide may represent SBP, whereas the faster migrating form corresponds to S64/SBP homologue. Consistent with this observation, the predicted M_r of SBP (60 552) is slightly higher than that of S64 (55 834). Thus, *SBP* and *S64* cDNAs may correspond to nonallelic *SBP* genes from soybean. Investigation of the genomic complexity of the *SBP* genes by Southern blot analysis revealed a pattern of cross-hybridizing bands consistent with the argument that SBP is encoded by a small gene family in soybean (data not shown). In view of this observation, the S64 protein may also be designated SBP2 (isoform 2), whereas the previously identified SBP [9] would be SBP1 (isoform 1).

The S64/SBP2 protein is a membrane-associated GTP-binding protein

The S64/SBP2 deduced protein contains a predicted nucleotide-binding site (Fig. 1) that harbors classical Walker-type consensus sequence for the P-loop, [Ala/Gly]x(4)Gly-Lys[Ser/Thr] [32]. Despite the fact that the

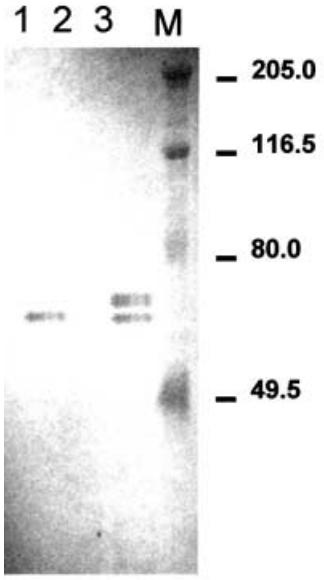

Fig. 3. Transient expression of S64 in cultured cotyledon cells. A plant expression vector containing *S64* cDNA under the control of 35SCaMV promoter and 3′ end of *rbcS* gene was electroporated into cultured soybean cells. Total protein from electroporated cells (1) and control cells (2) was extracted 2 days postelectroporation, fractionated by SDS/PAGE and immunoblotted with an anti-S64 serum. Lane 3 is a microsome preparation from 20 DAF seeds. M corresponds to prestaining molecular mass standards indicated on the right in kDa.

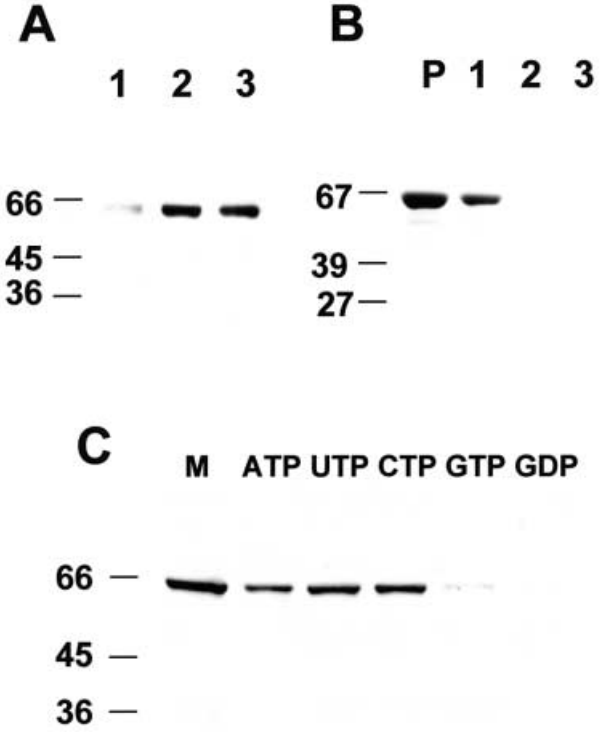

Fig. 4. Binding of S64/SBP homologue to nucleotides. (A) The S64/SBP2 protein cosediments with a GTP-agarose resin. Whole cell protein extracts from cultured soybean cells either nontransformed (1) or transiently transformed with *S64* cDNA expression construct (2) and transgenic tobacco cells expressing the soybean *S64* transgene (3) were incubated with GTP-agarose for 12 h. After rinsing the beads, bound proteins were solubilized in Laemmli sample buffer and separated on a 10% SDS/PAGE gel under reducing conditions. After transferring to nitrocellulose, proteins were detected with antibodies to S64/SBP2. The migration positions of molecular mass standards are indicated on the left in kDa. (B) Nucleotide binding assay of His-tagged S64/SBP2 protein. Purified His-S64 protein was incubated with GTP-agarose (1), ATP-agarose (2) or Protein A-agarose (3) resins for 1 h at 4 °C. The resins were washed with binding buffer and the bound proteins were eluted by SDS/PAGE sample buffer at 100 °C. Samples were analyzed by SDS/PAGE and Coomassie Blue staining. Lane P corresponds to the purified His-tagged S64 fusion protein. The migration positions of molecular mass standards are indicated on the left in kDa. (C) GTP binding of S64/SBP2 in the presence of various nucleotides. Purified His-S64 protein was incubated with binding buffer containing 2 mM competitor nucleotides, as indicated on the top of the figure, for 30 min at 4 °C prior to the addition of GTP-agarose. Resin-bound proteins were eluted as in (B), separated by SDS/PAGE and immunoblotted using an anti-S64 serum. In lane M, the GTP-agarose binding assay was performed in the absence of nucleotide competitor. The migration positions of molecular mass standards are indicated on the left in kDa.

sequence Ala-Leu-Ala-Pro-Thr-Lys-Lys-Ser (position 279–286) differs from the nucleotide binding consensus sequence in the sixth position where a Lys replaces the conserved Gly, we tested whether S64/SBP2 had the capacity to bind GTP. Whole-cell protein extracts obtained from cultured soybean cells transiently transformed with *S64*/*SBP2* cDNA under the control of the 35S promoter and from transgenic tobacco (*Nicotiana tabacum* L. Cv Havana) cell lines expressing soybean *S64*/*SBP2* transgene [21] were allowed to bind to GTP-agarose overnight. The bound proteins were then recovered from the pelleted beads by boiling in SDS-sample buffer and analyzed by SDS/PAGE followed by immunoblotting with antibodies to S64/SBP2 (Fig. 4A). The recombinant protein synthesized in transiently transformed soybean cells and in transgenic tobacco cell lines bound to GTP-agarose (lanes 2 and 3). In control cultured soybean cells, the endogenous protein was also detected at 55–58 kDa in immunoblottings of GTP-agarose precipitates (lane 1). The anti-S64 Ig cross-reactive protein seemed to be specifically associated with GTP, as it was not selected in control precipitates with agarose resin alone (data not shown). The electrophoretic mobility of the anti-S64 Ig cross-reactive GTP-agarose bound protein together with its over-accumulation in transgenic cell lines (compare lanes 2 and 3 with lane 1) suggested that S64/SBP2 bound GTP. Nevertheless, despite the presence of the nucleotide binding consensus motif in SBP sequence (Fig. 1), our data did not allow us to determine precisely if SBP (SBP1 isoform) also binds GTP. Although SBP is immunologically related to S64/SBP2, as the anti-S64 serum recognized both proteins in microsome preparation from soybean seeds, it is synthesized at very low levels in soybean suspension cells, and in the majority of our assays SBP accumulation was below the detection level. Attempts to increase the total protein extract as starting material in the binding reaction led to a remarkable increase in the background levels compromising the quality and interpretation of the data.

The precipitation of S64/SBP2 by GTP-agarose beads could reflect either direct binding of the protein to GTP or

previous association of S64/SBP2 with GTP-binding proteins present in the whole cell extracts. To examine these possibilities, we analyzed the capacity of purified *E. coli*-expressed His-tagged S64/SBP2 fusion protein to bind GTP (Fig. 4B). A fraction of the starting material (lane 1) bound to agarose-immobilized GTP (lane 2), whereas protein binding to ATP-agarose resin was negligible (lane 3). The S64/SBP2 recombinant protein also did not bind to protein A-agarose resin (lane 3). The specificity of S64/SBP2 binding to guanine nucleotides was further confirmed in competition assays (Fig. 4C). Incubation of the recombinant protein with 100-fold molar excess of GTP (lane GTP), GDP (lane GDP) and GTPγS (data not shown) prior to the binding reaction prevented the recovery of a large fraction of the protein through GTP-agarose resin. In contrast, excess of ATP, UTP and CTP did not abolish S64/SBP2 binding to GTP-agarose resin. Taken together, our data indicate that S64/SBP homologue exhibited a high degree of selectivity to guanine nucleotides (GTP, GDP, GTPγS) over adenine and pyrimidine nucleotide triphosphates.

The GTP binding activity of *E. coli*-produced protein was also investigated by a filter-binding assay. Immobilized His-wild-type S64/SBP2 fusion protein efficiently binds GTP in the presence of 4 μM unlabelled ATP nonspecific competitor (Fig. 5B, lane N and 5C, lane 1). A purified truncated version of the protein, in which the putative signal peptide and 149 amino acid residues from the C-terminus were deleted (Fig. 5A, lane 2), retained the capacity to bind GTP (Fig. 5B, lane T), albeit not to wild-type levels. The putative GTP-binding site was further mapped by site-directed mutagenesis. Replacement of Thr283 and Lys284 residues with Leu and Glu residues prevented GTP binding (Fig. 5B, lane M; Fig. 5C, lane 2), indicating that these residues are critical for binding.

Although there was some variation on protein amount, the mutant recombinant protein and the fusion-truncated protein accumulated to similar levels in the heterologous expression system (Fig. 5A). Thus, the lack of GTP binding of the mutant protein was not due to a decrease in protein stability that could account for loss of protein integrity during the dot blot assay. Previous experiments have shown that SBP is organized *in vivo* as dimers and trimers whose subunits interact to each other through disulfide linkage [16]. The mutation on the GTP binding site should have no effect on oligomerization if the proteins are properly expressed and folded. To certify that the failure of the GTP binding mutant to bind GTP was not due to global misfolding, the mutant protein was assayed for its capacity to form oligomers under nonreducing conditions (Fig. 6). *E. coli*-produced wild-type, truncated and mutant proteins were purified and separated by electrophoresis in the presence (+2-mer) and absence (−2-mer) of 2-mercaptoethanol. Under reducing conditions (+2-mer), the wild-type (lane 1) and truncated protein (lane 2) migrated at M_r 55 000 and 44 000, respectively, that correspond to the predicted M_r of their monomeric forms. The removal of the 2-mercaptoethanol caused a large fraction of both proteins to migrate at an M_r approximately twice greater than the corresponding monomer (−2-mer, lanes 1 and 2). In addition, a fraction of both proteins migrated as larger complexes that may correspond to their trimeric forms. These results indicate that both wild-type S64/SBP2 and the truncated protein oligomerize as dimers and trimers, which are stabilized by disulfide bounds. The identity of the oligomers was confirmed by immunoblotting (data not shown). The electrophoretic pattern of the mutated protein in the presence and absence of the reducing agent was very similar to that observed for the wild-type protein (compare lanes 1 and 3). The presence of high molecular mass migrating forms of the mutated protein in the absence of 2-mercaptoethanol indicated that mutation in the GTP binding sequence did not impair the capacity of the protein to self-associate into dimers and trimers.

GTP binding is not required for S64/SBP2-mediated sucrose transport in yeast

Functional complementation assays using the engineered *susy*7 yeast have demonstrated that SBP mediates sucrose uptake across the plasma membrane [17]. The SBP-mediated sucrose transport in yeast has been characterized biochemically and displays nonsaturable, linear uptake

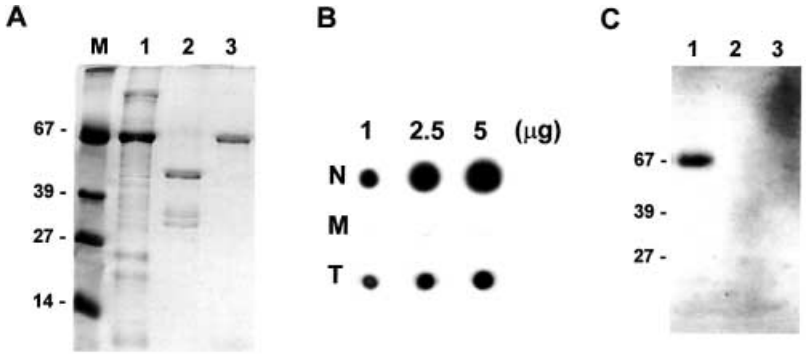

Fig. 5. GTP-binding of S64/SBP2. (A) N-Terminal His-tagged S64 fusion protein of the wild-type (His-S64) construct (1), truncated His-S64 protein (2) and Thr238Leu, Lys284Glu mutant His-S64 protein (3) were produced in *E. coli*, affinity-purified and separated by SDS/PAGE. Molecular mass markers (kDa) are shown on the left. (B) Increasing amounts (1, 2.5, and 5 μg) of *E. coli*-produced wild-type recombinant protein (N), Thr238Leu, Lys284Glu mutant protein (M) and truncated protein (T) were blotted onto nitrocellulose and reacted with [α-^{32}P]-GTP as described in methods. (C) Affinity-purified recombinant proteins produced in *E. coli* were separated by SDS/PAGE, electroblotted onto nitrocellulose and reacted with [α-^{32}P]-GTP. Lane 1 corresponds to affinity-purified wild type fusion protein, lane 2 to Thr238Leu, Lys284Glu mutant recombinant protein and lane 3 to an unrelated control protein (BSA).

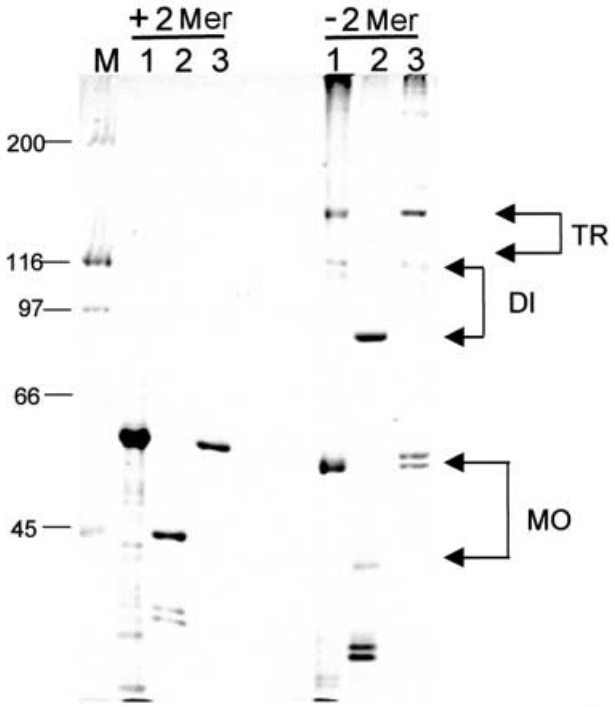

Fig. 6. Oligomerization of *E coli*-produced S64/SBP2 proteins. *E. coli*-produced wild-type recombinant protein (1), truncated protein (2) and mutant protein (3) were affinity-purified, treated with Factor Xa, solubilized in sample buffer prepared with (+2-Mer) and without (−2-Mer) 2-mercaptoethanol and separated on a 8% SDS/polyacrylamide gel. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue. Molecular mass standards are shown in M and their units are in kDa. TR denotes trimer; DI, dimer and MO, monomer.

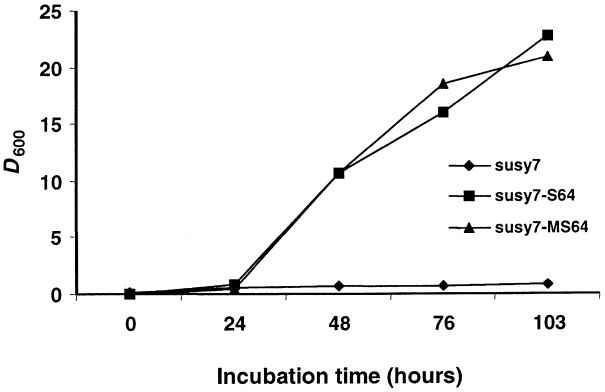

Fig. 7. Relative growth rate of susy7 transformed with either the wild type cDNA construct (susy7-S64) or the mutated cDNA construct (susy7-MS64) on sucrose-based medium. About 200 μL of a 24-h-old liquid culture of *susy*7 alone, *susy*7-S64 or *susy*7-MS64 growing in complete medium with 2% (w/v) glucose were used to inoculate 20 mL of yeast complete medium supplemented with 2% (w/v) sucrose. D_{600} were taken on 1 mL aliquots at the indicated incubation time.

kinetics [15]. Heterologous expression of S64/SBP2 in *susy*7 yeast, which is deficient in utilizing extracellular sucrose, restored the ability of this strain to grow on sucrose as the sole carbon source, providing evidence that S64 and SBP are functionally analogs (Fig. 7). To determine the effect of the GTP binding site mutations on S64/SBP2-mediated sucrose transport, we assayed the capacity of these mutants to promote growth of the *susy*7 yeast strain on sucrose medium. The *susy*7-S64 yeast and *susy*7-MS64 (transformed with site-directed mutant *S64* cDNA) displayed similar growth rate when transferred to a medium supplemented with sucrose as the only carbon source. Thus, the site-directed mutant failed to bind GTP but not to mediate sucrose transport in yeast.

DISCUSSION

The sucrose binding protein is a membrane-associated protein that has been shown to mediate sucrose transport in yeast [15,17]. We showed that a member of this family, designated S64 or SBP2, binds GTP, although it is not structurally related to other GTP binding proteins. The heterotrimeric and monomeric G-proteins contain four consensus GTP-binding motifs [A/GXXXXGK(S/T), DXXG, NXXG, and (C/S)AX] that fold into a structurally conserved GTP-binding site comprised of five α helices and a central six-stranded β sheets [33,34]. In contrast, the sucrose binding protein shows no structural homology with other GTP-binding proteins, except for the presence of an imperfect nucleotide binding A consensus Ala279-X-X-X-X-Lys-Lys-Ser286, in which the conserved Gly is replaced by a Lys284, and a second GTP binding motif D376-X-X-G379. In the Walker-type A consensus sequence or P loop, the loop formed between a β strand and an α helix interacts with the phosphate groups of the nucleotide. In the S64/SBP homologue, the single deviation (Gly/Lys) from the pattern seems not to interfere with the nucleotide binding because we have mapped by site-directed mutagenesis the GTP binding site of S64/SBP2 to this imperfect A consensus sequence. It is very likely that the positive charge of the Lys284 residue could be involved in potential electrostatic interactions between negatively charged phosphate groups of GTP, as the introduction of negatively charged Glu (Lys284Glu) residue abolished GTP binding (Fig. 5). Alternatively, the imperfect Walker-type A consensus sequence may not contribute specific contacts with GTP but, instead, mutations in this sequence caused misfolding of the protein, thereby affecting multiple functions. This possibility was raised because the specific amino-acid replacements made in the putative binding site (Thr283Leu and Lys284Glu changes) do not represent structurally neutral changes. However, two lines of evidence suggest that incorrect folding cannot account for the failure of the mutant protein to bind GTP. First, the site-directed mutations did not impair S64/SBP2-mediated sucrose transport, implying that the mutant protein retains the capacity to bind sucrose and to assemble correctly with the plasma membrane (Fig. 7). Second, mutation did not prevent S64/SBP2 oligomerization, further indicating that their effects were specific for GTP binding (Fig. 6). Although definitive evidence for involvement of this sequence in GTP binding will require further studies, including the determination of the crystal structure of GTP-bound S64/SBP2, the characterization of the site-directed mutant presented here is consistent with the direct involvement of the Lys284 residue in binding GTP.

We also analyzed a possible role of the GTP-binding activity of SBP2 on its capacity to transport sucrose in a yeast deficient invertase strain. Complementation assays in the mutant yeast strain incapable in utilizing extracellular sucrose demonstrated that the GTP binding site of SBP2 was not essential for the sucrose transporting capacity mediated by heterologous overexpression of the protein. This result may indicate that the GTP binding and transport activities of S64/SBP2 are separable and can function independently.

Consistent with its function as a transport protein, topological characterization of SBP in purified plasma membrane vesicles has demonstrated that a proportion (25%) of SBP behaves as a type II membrane protein, which spans the bilayer once and has the bulk of the protein exposed to the extracellular environment [10]. The remaining protein (about 75%) is a peripheral associated-membrane protein. The fraction of intrinsic membrane protein has been proposed to be tethered to the plasma membrane by its uncleavable putative N-terminal leader peptide, the only hydrophobic region of the protein that could function as a membrane-spanning domain (Fig. 1). Consistent with this hypothesis, the leader peptide is not quantitatively cleaved from *in vitro*-transcribed and -translated SBP and S64/SBP2 in the presence of microsome ([10]; data not shown). According to this topological model, the oligomerization properties of the protein would provide the means for assembling protein conduits across the membrane to mediate sucrose transport. This possibility has been previously considered with the observation that SBP is structurally related to vicilin-like storage proteins [16,35], which assemble into trimers to form a 86–88 Å toroid complex with an internal hole of 18 Å [36,37]. Conserved residues that are involved in stabilizing the three-dimensional structure of vicilin-like proteins are found at similar positions in the SBP and S64 sequence (Fig. 1). As S64 and SBP share an extraordinary conservation of primary structure that extends to include identical tertiary motifs, it is very likely that the S64/SBP2 protein might also fit into the canonical model proposed for the structure of the vicilin-like protein family.

The proposed topology for SBP/S64, as an intrinsic membrane transporter, would also predict that GTP binding might not affect sucrose transport as nucleotides are absent extracellularly. A consequence of this hypothesis is that S64/SBP2-mediated sucrose transport functions independently of its GTP binding activity (Fig. 7). In contrast, any biological significance for the presence of a functional GTP binding site would be strictly dependent on the intracellular localization of the protein where high nucleotide concentration is present. While previous result based on NHS-biotin labeling membrane proteins demonstrated that SBP was associated with the external surface of the membrane [10], they did not exclude other sites of cellular localization that would provide the appropriate compartmentalization for a functional GTP binding properties. Recently, a *Vicia faba* SBP-like protein (*Vf*SBPL) was found to accumulate predominantly in the protein storage vacuole, but a small fraction of the protein was also detected in the plasma membrane of cotyledonary cells [38]. One possibility is that a proportion of the soybean SBP is indeed localized extracellularly at the cell surface as a type II membrane transport protein and a fraction of correctly processed protein remains intracellularly as membrane-associated protein where its capacity to bind GTP and sucrose may implicate a regulatory role. Further experiments will be necessary to confirm the proposed topology for SBP and its subcellular localization.

Many studies in plants have described sugar-mediated changes in gene expression and recent research has provided convincing evidence for a sucrose-dependent signaling pathway, as an important regulatory step in resource allocation [1,39,40]. The demonstration that S64/SBP2 exhibits GTP binding activity together with its capacity to bind sucrose specifically and reversibly [8] raises the possibility that SBP may also serve a regulatory role in sucrose translocation-dependent physiological processes in plants. The resulting phenotypes from alteration on SBP levels in transgenic plants may support such function [14,21]. S64/SBP repression studies in tobacco have indeed shown some of the typical phenotypes caused by impairment of sucrose translocation [4,41,42], such as accumulation of carbohydrates within source leaves, inhibition of photosynthesis and stunted growth [14]. Nevertheless, the pattern of sugar accumulation in the S64 antisense leaves was not identical to that caused by antisense repression of H^+/SUT1 symporter [4]. This observation suggests that SBP and SUT have distinct functions in sucrose translocation and favors the argument that SBP serves a regulatory role in the plant sucrose uptake system. Consistent with this hypothesis, manipulation of S64/SBP levels in transgenic plants and cultured cells correlated inversely with cell-wall invertase activity and directly with sucrose synthase activity [14,21]. Remarkably, the increase in S64/SBP levels had a stronger effect on sucrose synthase activity than on sucrose uptake [22]. These observations further support the idea that S64/SBP functions in the sucrose translocation pathway by regulating the expression or activity of alternative carbohydrate uptake systems. In addition, they may provide an alternative explanation for the capacity of SBP to promote growth of *susy7* yeast on sucrose as a sole carbon source, as this strain harbors a potato sucrose synthase gene integrated into its genome [3]. Thus, the functional complementation assays in *susy7* yeast for sucrose transport processes have been conducted in the presence of an intracellular plant sucrose cleaving activity. Further studies will be necessary to discern whether S64/SBP2 mediates sucrose transport or functions directly as regulator of sucrose metabolizing enzymes or both.

The importance of the present study is twofold. First, we have localized residues required for GTP-binding by SBP and have shown that this binding probably involves a novel GTP-binding folding protein. Second, this is the first report of a sucrose binding protein that also binds GTP. The demonstration that S64/SBP2 is a GTP binding protein may provide new insight into the role of this protein in sucrose translocation-dependent physiological processes in plants. Nonetheless, much remains to be learned about many aspects of post-translational modification and regulation of SBP and even the most general aspects of SBP-mediated transport in plants. The specific mutant can now be used to dissect the physiological role of SBP as a G-protein in transgenic plants and cultured cells.

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REFERENCES

1. Lalonde, S.L., Boles, E., Hellmann, H., Barker, L., Patrick, J.W., Frommer, W.B. & Ward, J.M. (1999) The dual function of sugar carriers: transport and sugar sensing. *Plant Cell* **11**, 707–726.
2. Delrot, S. & Bonnemain, J.-L. (1981) Involvement of protons as a substrate for the sucrose carrier during phloem loading in *Vicia faba* leaves. *Plant Physiol.* **67**, 560–564.
3. Riesmeier, J.W., Willmitzer, L. & Frommer, W.B. (1992) Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast. *EMBO J.* **11**, 4705–4713.
4. Riesmeier, J.W., Willmitzer, L. & Frommer, W.B. (1994) Evidence for an essential role of the sucrose transporter in phloem loading and assimilate partitioning. *EMBO J.* **13**, 1–7.
5. Bürkle, L., Hibberd, J.M., Quick, W.P., Kühn, C., Hirner, B. & Frommer, W.B. (1998) The H^+-sucrose cotransporter NtSUT1 is essential for sugar export from tobacco leaves. *Plant Physiol.* **118**, 59–68.
6. Weise, A., Barker, L., Kühn, C., Lalonde, S., Buschmann, H., Frommer, W.B. & Ward, J.M. (2000) A new subfamily of sucrose transporters, SUT4, with low affinity/high capacity ocalized in enucleate sieve elements of plants. *Plant Cell* **12**, 1345–1355.
7. Barker, L., Kühn, C., Weise, A., Schulz, A., Gebhardt, C., Hirner, B., Hellmann, H., Schulze, W., Ward, J.M. & Frommer, W.B. (2000) SUT2, a putative sucrose sensor in sieve elements. *Plant Cell* **12**, 1153–1164.
8. Ripp, K.G., Viitanen, P.V., Hitz, W.D. & Franceschi, V.R. (1988) Identification of a membrane protein associated with sucrose transport into cells of developing soybean cotyledons. *Plant Physiol.* **88**, 1435–1445.
9. Grimes, H.D., Overvoorde, P.J. & Hipp, K. (1992) A 62-kD sucrose binding protein is expressed and localized in tissues actively engaged in sucrose transport. *Plant Cell* **4**, 1561–1574.
10. Overvoorde, P.J. & Grimes, H.D. (1994) Topographical analysis of the plasma membrane-associated sucrose binding protein from soybean. *J. Biol. Chem.* **269**, 15154–15161.
11. Warmbrodt, R.D., Buckhout, T.J. & Hitz, W.D. (1989) Localization of a protein, immunologically similar to a sucrose-binding protein from developing soybean cotyledons, on the plasma membrane of sieve-tube members of spinach leaves. *Planta* **180**, 105–115.
12. Warmbrodt, R.D., Vanderwoude, W.J. & Hitz, W.D. (1991) Studies on the localization of a protein, immunologically similar to a 62-kilodalton sucrose-binding protein isolated from developing soybean cotyledons, in the shoot and root of spinach. *New Phytol.* **118**, 501–511.
13. Harrington, G.N., Franceschi, V.R., Offler, C.E., Patrick, J.W., Tegeder, M., Frommer, W.B., Harper, J.F. & Hitz, W.D. (1997) Cell specific expression of three genes involved in plasma membrane sucrose transport in developing *Vicia faba* seed. *Protoplasma* **197**, 160–173.
14. Pedra, J.H.F., Delú-Filho, N., Pirovani, C.P., Contim, L.A.S., Dewey, R.E., Otoni, W.C. & Fontes, E.P.B. (2000) Antisense and sense expression of a sucrose binding protein homologue gene from soybean in transgenic tobacco affects plant growth and carbohydrate partitioning in leaves. *Plant Sci.* **152**, 87–98.
15. Overvoorde, P.J., Frommer, W.B. & Spencer, D. (1996) A soybean sucrose binding protein independently mediates nonsaturable sucrose uptake in yeast. *Plant Cell* **8**, 271–280.
16. Overvoorde, P.J., Chao, W.S. & Grimes, H.D. (1997) A plasma membrane sucrose-binding protein that mediates sucrose uptake shares structural and sequence similarity with seed storage proteins but remains functionally distinct. *J. Biol. Chem.* **272**, 15898–15904.
17. Grimes, H.D. & Overvoorde, P.J. (1996) Functional characterization of sucrose binding protein-mediated sucrose uptake in yeast. *J. Exp. Bot.* **47**, 1217–1222.
18. Lin, W., Schmitt, M.R., Hitz, W.D. & Giaquinta, R.T. (1984) Sugar transport into protoplast isolated from developing soybean cotyledons. *Plant Physiol.* **75**, 936–940.
19. Maynard, J.W. & Lucas, W.J. (1982a) A re-analysis of the two-component phloem loading system in *Beta vulgaris*. *Plant Physiol.* **69**, 734–739.
20. Maynard, J.W. & Lucas, W.J. (1982b) Sucrose and glucose uptake into Beta vulgaris leaf tissues. *Plant Physiol.* **70**, 1436–1443.
21. Delú-Filho, N., Pirovani, C.P., Pedra, J.H.F., Matrangolo, F.S.V., Macêdo, J.N.A., Otoni, W.C. & Fontes, E.P.B. (2000) A sucrose binding protein homologue from soybean affects sucrose uptake in transgenic tobacco suspension-cultured cells. *Plant Physiol. Bioch.* **38**, 353–361.
22. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2th edn. Cold Spring. Harbor Laboratory Press, Cold Spring Harbor, NY.
23. Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.
24. Cascardo, J.C.M., Almeida, R.S., Buzeli, R.A.A., Carolino, S.M.B., Otoni, W.C. & Fontes, E.P.B. (2000) The phosphorylation state and expression of soybean BiP isoforms are differentially regulated following abiotic stresses. *J. Biol. Chem.* **275**, 14494–14500.
25. Fontes, E.P.B., Eagle, P., Sipes, P. & Hanley-Bowdoin, L. (1994) Interaction between a gemini virus replication protein and origin DNA is essential for replication. *J. Biol. Chem.* **269**, 8459–8465.
26. Alvim, F.C., Carolino, S.M.B., Cascardo, J.C.M., Nunes, C.C., Martinez, C.A., Otoni, W.C. & Fontes, E.P.B. (2001) Enhanced accumulation of BiP in transgenic plants confers tolerance to water stress. *Plant Physiol.* **126**, 1042–1054.
27. Murashige, T. & Skoog. F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* **15**, 473–497.
28. Laemmli, U.K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
29. Gaudry, C.A., Verderio, E., Aeschlimann, D., Cox, A., Smith, C. & Griffin, M. (1999) Cell surface localization of tissue transglutaminase is dependent on a fibronectin-binding site in its N-terminal β-sandwich domain. *J. Biol. Chem.* **274**, 30707–30714.
30. Wagner, P., Hengst, L. & Gallwitz, D. (1992) Ypt proteins in yeast. *Methds Enzymol.* **153**, 3–11.
31. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. & Struhl, K. (1994) Basic local alignment search tool. *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc, New York.
32. Saraste, M., Sibbald, P.R. & Wittinghofer, A. (1990) The P-loop – a common motif in ATP- and GTP-binding proteins. *Trends Biochem. Sci.* **15**, 430–444.
33. Hamm, H.E. (1998) The many faces of G protein signaling. *J. Biol. Chem.* **273**, 669–672.
34. Stenmark, H. & Olkkonen, V.M. (2001) The Rab GTPase family. *Genome Biol.* **2**, 3007.1–3007.3007.7.

35. Braun, H., Czihal, A., Shutov, A.D. & Bäumlein, H. (1996) A vicilin-like seed protein of cycads: similarity to sucrose binding proteins. *Plant Mol. Biol.* **31**, 35–44.
36. Ko, T.P., Ng, J.D. & mcPherson, A. (1993) The three-dimensional structure of canavalin from jack bean (*Canavalia ensiformis*). *Plant Physiol.* **101**, 729–744.
37. Lawrence, M.C., Izard, T., Beuchat, M., Blagrove, R.J. & Colman, P.M. (1994) Structure of phaseolin at 2.2 Å Resolution. *J. Mol. Biol.* **238**, 748–776.
38. Heim, U., Wang, Q., Kurz, T., Borisjuk, L., Golombek, S., Neubohn, B., Adler, K., Gahrtz, M., Sauer, N., Weber, H. & Wobus, U. (2001) Expression patterns and subcellular localization of a 55 kDa sucrose-binding protein homologue of *Vicia faba* (VfSBPL) suggest different functions during development. *Plant Mol. Biol.* **47**, 461–474.
39. Gibson, S.I. (2000) Plant sugar- response pathways. Part of a complex regulatory web. *Plant Physiol.* **124**, 1532–1539.
40. Smeekens, S. (2000) Sugar-induced signal transduction in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51**, 49–81.
41. Kühn, C., Quick, W.P., Schulz, A., Sonnewald, U. & Frommer, W.B. (1996) Companion cell-specific inhibition of the potato sucrose transporter SUT1. *Plant Cell Environ. Plant Cell Environ.* **19**, 1115–1123.
42. Lemoine, R., Kühn, C., Thiele, N., Delrot, S. & Frommer, W.B. (1996) Antisense inhibition of the sucrose transporter in potato: effects on amount and stability. *Plant Cell Environ.* **19**, 1124–1131.