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Article

Mutational Analysis of the Effector Domain of *Brassica* Sar1 Protein

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Sar1p is a ras-related GTP-binding protein that functions in intracellular protein transport between the endoplasmic reticulum (ER) and the Golgi complex. The effector domain of Ras family proteins is highly conserved and this domain is functionally interchangeable in plant, yeast and mammalian Sar1. Using a recombinant Brassica sar1 protein (Bsar1p) harboring point mutations in its effector domain, we here investigated the ability of Sar1p to bind and hydrolyze GTP and to interact with the two sar1-specific regulators, GTPase activating protein (GAP) and guanine exchange factor (GEF). The T51A and T55A mutations impaired Bsar1p intrinsic GTP-binding and GDP-dissociation activity. In contrast, mutations in the switch domain of Bsar1 did not affect its intrinsic GTPase activity. Moreover, the P50A, P54A, and S56A mutations affected the interaction between Bsar1p and GAP. P54A mutant protein did not interact with two regulating proteins, GEF and GAP, even though the mutation didn't affect the intrinsic GTP-binding, nucleotide exchange or GTPase activity of Bsar1p.

Key words: *effector domain, GAP, GEF, Mutagenesis, Sar1 GTPase*

The protein secretory machinery is highly conserved in eukaryotic cells. The secretory pathway starts in the ER, where newly synthesized proteins undergo folding, maturation and oligomerization [Hurtley *et al.*, 1989]. In the Golgi apparatus, the proteins are further processed and sorted for delivery to their final destinations [Palade, 1975].

The transport of proteins from the ER to their target sites requires the formation of protein vesicles. This process involves the stepwise recruitment of the small GTPase, Sar1p, and two large heterodimeric complexes of Sec23-Sec24 and Sec13-Sec31 to the ER membrane. Within this protein complex, Sar1p tightly interacts with the GTPase activating protein (GAP), Sec23. During trafficking, COPI vesicles are known to be involved in both anterograde and retrograde transport, whereas COPII-coated vesicles are responsible for anterograde cargo transport [Fromme *et al.*, 2005]. To initiate COPII coat assembly at ER exit sites, Sec12p, an ER transmembrane protein, acts as a guanine exchange factor (GEF) for Sar1 and catalyses the exchange of GTP for GDP on Sar1 [Fromme *et al.*, 2005]. Thus, the sequential action of COPI and COPII coat complexes is a widely accepted model for ER-to-Golgi transport.

The cDNAs encoding plant homologues of *Sar1*, *Sec12*, *Sec13* and *Sec23* have been cloned from plant sources and shown to associate with the ER [Bar-Peled *et al.*, 1997; Movafeghi *et al.*,1999; Kim *et al.*,1997; Takeuchi *et al.*, 2000]. Transient expression of a dominant negative mutant (AtSar1-H74L) and overexpression of Sec12p blocked the transport of a target protein from the ER to the Golgi apparatus in plant cells [Takeuchi *et al.*, 2000; Memon, 2004]. *In vitro* reconstitution experiments suggest that the same protein machinery (Sar1, Sec12, Sec23/24, Sec13/31) is also used in plant cells for anterograde transport from the ER to Golgi, even though COPII-coated vesicles have not been isolated or identified *in planta*.

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Abbreviations: ER, Endoplasmic Reticulum; GAP, GTPase Activating Protein; GEF, Guanine Exchange Factor

Sar1p is a ras-related, small GTP-binding protein. It contains two switch regions (I and II) that are located at proximal sites to their dimeric interface and it interacts with hydrolyzing factors as well as effector molecules of guanine nucleotide exchange [Vetter *et al.*, 2001]. Despite several previous biochemical studies of sar1p in yeast and mammals, there have been very few reports on the role(s) of its effector domain in plant cells. Structural alignment of Bsar1p with other subfamilies of small GTPases, however, facilitated the identification of the two switch regions of Bsar1p, that correspond to amino acid residues 45-56 (switch I) and 75-91 (switch II) [Kim *et al.*, 1997]. Therefore, in this paper, we clarified the functional importance of the Bsar1p effector domain by individually mutating the five highly conserved amino acids (P50, T51, P54, T55, and S56) in this region. Using bacterially expressed recombinant Bsar1 proteins, we measured the effects of these mutations on their GTP-binding, GDP-dissociation, and GTPase activities. In addition, we also tested the effect of each amino acid substitution on the interaction of BSar1p with two regulatory molecules, Sec23GAP and Sec12GEF, which play a pivotal role in protein secretion from the ER to Golgi. Using this approach we demonstrated that each conserved residue contributes differently to the activities of BSar1p, and that proline at position 54 is important for the interaction of BSar1p with Sec23-GAP and Sec12-GEF.

Materials and Methods

U-DNA mutagenesis. For point mutation of wild-type (WT) *Bsar1* cDNAs, we used the U-DNA mutagenesis method [Kunkel *et al.*, 1985]. To prepare a single-stranded DNA template, the pBluescript SK$^-$-*Bsar1a* was introduced into the *Escherichia coli* strain, CJ236. Single-stranded DNA was obtained after infection with helper phage VCSM13 (Stratagene). The nucleotide sequences used to generate point mutations were as follows:

P50A; 5'-TGCAGCACCAGGCGACGCAGCATC-3',
T51A; 5'-TGCAGCACCAGCCGGCGCAGCATC-3',
P54A; 5'-ACGCAGCATGCAACATCTGAAGAA-3',
T55A; 5'-ACGCAACATGCAGCATCTGAAGAA-3',
S56A; 5'-CATGCAACAGCTGAAGAACTAAGC-3'

The accuracy of mutagenesis was verified by DNA nucleotide sequencing.

Expression and purification of WT and recombinant GST-Bsar1 proteins. WT and mutant *Bsar1* cDNAs were cloned into the *pGEX* expression vector. GST-Bsar1 proteins were expressed in *E. coli BL21(DE3)* and purified with glutathione-agarose (Sigma, St. Louis, MO), as described previously [Jang *et al.*, 2004].

Guanidine nucleotide binding assay. The GTP overlay assay was performed according to Kim *et al.* (1999) with slight modifications. Purified GST-Bsar1 proteins were separated on a 10% SDS-PAGE gel and electrophoretically transferred to a PVDF membrane (immobilon-P; Millipore, MA, USA). The membrane was incubated in a binding buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.2% Tween 20, 0.25 mg BSA and 0.5 mM EDTA) containing 0.1 mM [α-^{32}P]GTP (3,000 Ci/mM; ICN) for 1 hr and developed using Kodak X-ray film.

Assay of GDP dissociation rate. A filter binding assay was performed essentially as described [Zhang *et al.*, 1998]. GST-Bsar1 protein was reacted with [^{3}H]GDP in buffer A (50 mM HEPES, pH 7.6, 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT) containing 10 mM EDTA, at 25°C for 30 min. Following incubation, 5 mM $MgCl_2$ was added to the reaction product and the mixture was incubated on ice for 10 min. The unbound [^{3}H]GDP was removed by rapid gel filtration. The dissociation reaction was initiated by adding excess GTP and was carried out in buffer A. At constant time intervals, aliquots of the mixture were withdrawn, filtered and washed with cold stop solution. Radioactivity retained in the GST-Bsar1p was measured by liquid scintillation counting.

GTPase activity assay. To quantify intrinsic GTPase activity, 500 ng of GST-Bsar1 was incubated at 30°C in HEDL buffer containing 0.2 μM [γ-^{32}P]GTP and 5 mM $MgCl_2$. Time-dependent release of ^{32}Pi was measured upon the addition of GST-Bsar1. At indicated time points, 25-μL aliquots were removed and 775 μL of 5% (w/v) charcoal in ice-cold 50 mM NaH_2PO_4 was added. After removal of charcoal by centrifugation, total radioactivity in a 400-μL aliquot of the supernatant was measured [Brandit *et al.*, 1985].

Sec23 protein purification and assay of its GTPase activating activity. The cytosolic fraction of RSY255 (*Ura 3-52, Leu 2-3, 112, MAT*α transformed with *pCF23)* was used to purify Sec23p. This was conducted by sequential chromatography on DEAE Sepharose and S-Sepharose Fast Flow columns. Sec23p was detected by Western blotting with an antibody raised against it [Hicke *et al.*, 1992]. The GTPase activating activity of purified Sec23p was determined according to the method described by Wagner *et al.*, 1987.

Complementation of temperature-sensitive (ts) mutant phenotype of *sec12-1* yeast. Functional complementation of the *ts*-mutant phenotype of *sec12-1* yeast was carried out using WT and mutant Bsar1 genes ligated into the yeast expression vector, *pVT-U.* The plasmids were transformed into the *sec12-1* mutant yeast by the lithium-acetate method [Takeuchi *et al.*, 2000; Zhang B *et al.*,

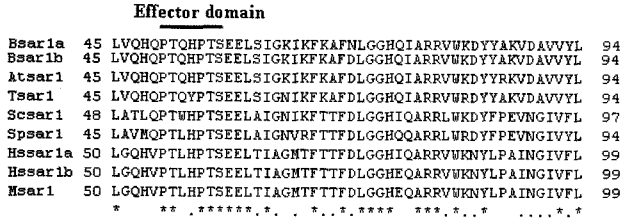

Fig. 1. Point-mutation of the Bsar1 protein within its putative effector domain. Amino acid sequences of the effector domain of Sar1 proteins in *B. campestris* (Bsar1a and Bsar1b), *A. thaliana* and *S. pombe* (Atsar1 and Spsar1), tomato (Tsar1), *S. cerevisiae* (Scsar1), human Cho cells (Hs-sar1a and Hs-sar1b), and mouse cells (Msar1) are aligned for comparison. Asterisks (*) denote positions perfectly conserved and dots (.) mark well-conserved positions. Amino acid substitutions in the effector domain are represented at the lower panel. P;proline, T; threonine, L; leucine, H; histidine, S; serine, A; alanine.

1998], and the resulting transformants were tested for temperature sensitivity, either at 25°C or 37°C.

Results and Discussion

Effect of point mutation in the effector domain of Bsar1p on GTP-binding, GDP-dissociation and GTPase activities. To analyze the functional importance of the Bsar1p effector domain, five highly conserved amino acids in this domain were individually changed by site-directed alanine substitution. The mutations were denoted P50A, T51A, P54A, T55A, and S56A, whereby P50A represents the substitution of proline at position 50 of Bsar1p to alanine etc. (Fig. 1). The bacterially expressed mutant proteins fused with GST were homogeneously purified (data not shown). Using these recombinant mutant proteins, we carried out a GTP-binding overlay assay to determine the effects of the individual mutations on Bsar1p GTP-binding ability. In comparison to WT Bsar1p, the P50A, P54A, and S56A mutants showed no difference (Fig. 2) in GTP-binding, suggesting that these amino acid substitutions induced no significant structural changes in Bsar1. In contrast, the T51A and T55A mutant proteins had greatly diminished GTP-binding activity (Fig. 2), indicating that these amino acids are critical for the interaction of Bsar1p with the γ-phosphate group of GTP. The crystal structure of yeast Sar1-GppNHp has already provided evidence that Thr51 in the switch 1 domain of BSar1p is critical for hydrogen bond formation between the γ-phosphate and Mg^{2+} in Sar1p-GppNHp [Bi

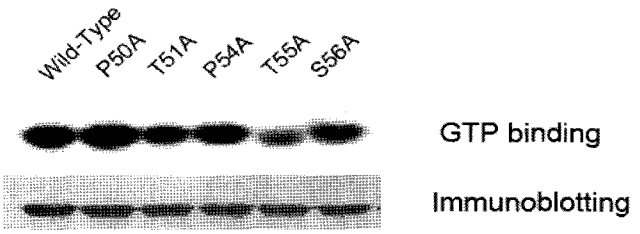

Fig. 2. GTP-binding activity of wild-type and point-mutated Bar1p proteins. Purified GST-fused Bsar1 proteins from *E.coli* were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with [α-^{32}P]GTP and autoradiographed (upper panel). Another equivalent membrane was subjected to Western blotting with an anti-Bsar1p antibody (lower panel).

et al., 2002]. It is remarkable that the T51A Bsar1p mutant still exhibits at least 50% of the GTP-binding activity observed for the WT protein.

Since the GDP-bound forms of Ras-related proteins are inactive, with GDP release often being the rate-limiting step in their activation, the replacement of GDP by GTP constitutes an essential step in their activation [Takai *et al.*, 2001]. Furthermore, the spatial and temporal regulation of nucleotide exchange and GTP hydrolysis is critically important in ensuring that coat assembly coordinates with cargo selection and that coat disassembly coincides with vesicle fission [Balch *et al.*, 1994]. Therefore, we determined the dissociation rates of pre-bound GDP to Bsar1p by filter binding assay for both WT and mutant Bsar1p proteins (Fig. 3). Under the experimental conditions used here, the dissociation of GDP from Bsar1 proteins follows a single exponential curve, as expected for a single class of nucleotide-binding site. The dissociation rates of Bsar1 mutant proteins fell into three groups. Firstly, the GDP-dissociation rate for the Bsar1 mutant, S56A, did not change at all, relative to WT Bsar1p (Fig. 3). However, a second group of mutant Bsar1 proteins, namely T51A and T55A, exhibited a significant decrease in their GDP-dissociation rate. Finally, the last group, comprising the P50A and P54A mutants, showed a slight reduction in GDP-dissociation rate. The observed half-lives of the bound GDP for the three groups of Bsar1 mutants was 3.5 min, more than 15 min and more than 5.2 min, respectively. As expected, T51A and T55A showed a defect in GTP binding as well as GDP dissociation, which is consistent with the recent finding that the GTP-binding step is a prerequisite for GDP-dissociation and is the rate-limiting step in the GEF reaction [Zhang *et al.*, 2005]. In addition, with regard to GTPase activity, which we measured under steady state conditions, no significant difference in GTP-hydrolyzing activity was observed for any of the mutant Bsar1p proteins,

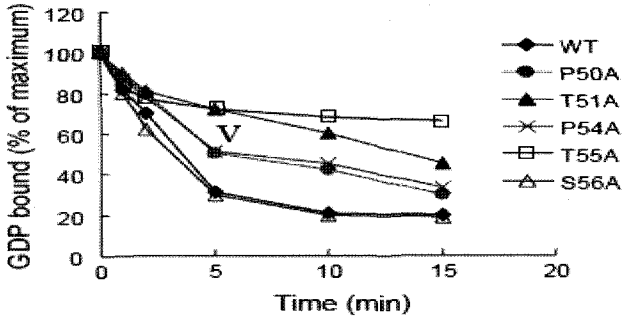

Fig. 3. Dissociation kinetics of GDP bound to various Bsar1 mutant proteins. Proteins were preincubated with 500 nM [^{3}H]GDP-labeled nucleotide for 20 min and chased with 1 mM cold nucleotide at 30°C. 100 μL of the reaction product was diluted with cold washing buffer and filtered through nitrocellulose at the indicated time points, following which radioisotopes remaining in the Bsar1 proteins were counted.

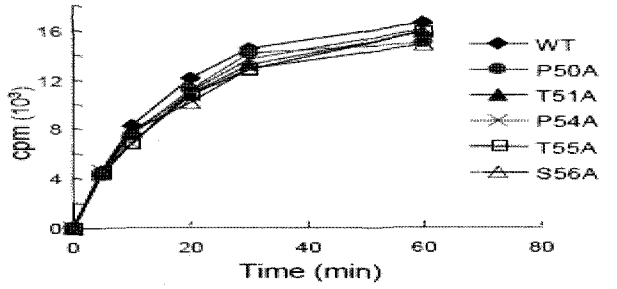

Fig. 4. Intrinsic GTPase activity of wild-type and mutant Bsar1 proteins. Steady-state GTPase activities were measured by the charcoal method as described in 'Materials and Methods'.

relative to WT (Fig. 4). These combined observations suggest that, contrary to Bsar1p GTP-binding affinity or GDP-dissociation rate, point mutation of the Bsar1 effector domain does not affect its intrinsic GTPase activity.

Effect of point-mutation of Bsar1p in its effector domain on Sec23p-mediated activation of Bsar1p GTPase activity. It has been shown that ER-to-Golgi SNAREs in yeast, as well as several integral membrane cargo proteins, directly bind to the pre-budding complex to increase their concentration in COPII vesicles [Miller *et al.*, 2003]. In this cargo-selection process, Sec23/24 plays a key role as a Sar1-GAP. Based on prior yeast and mammalian data indicating that mutations in the effector domain and in the conserved GTP-binding motif interfere with GAP-induced GTPase activity, we examined the effect of Sec23p, a partially purified yeast GAP, on the GTP-hydrolyzing activity of Bsar1 mutant proteins. Yeast Sec23p profoundly enhanced the GTPase activity of WT Bsar1p (Fig.5). However, the GTPase activity of the P54A mutant did not increase at all in the presence of

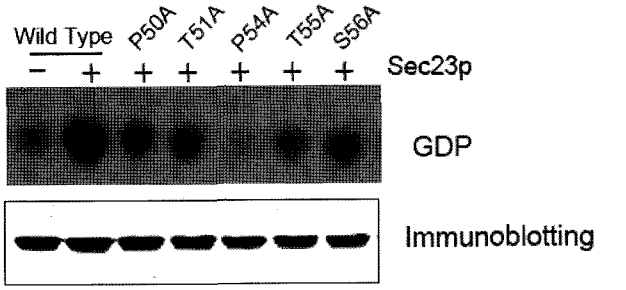

Fig. 5. Induction of Bsar1 protein GTPase activity by yeast Sec23p. GST-fused wild-type and mutant Bsar1p proteins were incubated in a reaction mixture with (+) or without (-) yeast Sec23p. GDP-bound GST-Bsar1 proteins were pulled down by GSH-sepharose gel. The nucleotide bound to Bsar1 proteins was recovered with EDTA and separated on a PEI-cellulose TLC plate. After developing the plate, it was exposed to X-ray film (upper panel). GST-fused wild-type and mutant proteins were separated on a 10 % SDS-PAGE gel and subjected to Western blotting with an anti-Bsar1p antibody (lower panel).

yeast Sec23p, whereas that of the other mutants, P50A, T51A, T55A, and S56A, was only slightly increased by Sec23p (Fig. 5). This result indicates that each of the five conserved amino acid residues in the switch I region of the Bsar1 effector domain is important for GTPase stimulation by Sec23p. It is in good agreement with results previously obtained for H-ras p21, which is regulated by human ras-GAP [Adari *et al.*,1988].

Complementation of temperature-sensitive (ts) *sec12-1* mutant yeast by the Bsar1 mutant proteins. *Sar1* was originally isolated as a multicopy suppressor of the *ts* Sec12p mutant yeast strain, which has defects in the ER-to-Golgi protein trafficking pathway [Nakano *et al.*, 1988]. The *sec12-1* mutant was identified as a variant harboring a P73L mutation in the cytosolic domain of Sec12p. The *Sec12-1* allele of the *Sec12* gene allows normal growth of yeast cells at 25°C but becomes lethal at 37°C [Barlowe *et al.*, 1993]. Elevation of *Sar1* gene dosage can suppress the *ts* defect of *sec12-1* but not the lethality of the *sec12* null mutant, suggesting that the direct interaction of the two gene products is a critical function for the ER-to-Golgi transport process. We have also reported that Bsar1a and Bsar1b are functional homologues of yeast Sar1p that can functionally complement the *ts sec12-1* mutant yeast [Kim *et al.*, 1999]. On the basis of these combined results, the ability of Bsar1 mutant proteins to suppress *ts sec12-1* mutant yeast lethality was investigated at restrictive temperatures. In contrast to WT and P50A and S56A mutant Bsar1 proteins, all of which rescued the *ts sec12-1* mutant phenotype, the other mutants, T51A, P54A, and T55A, were not able to complement *sec12-1* temperature-

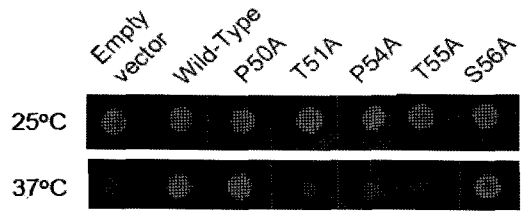

Fig. 6. Complementation of the temperature-sensitive (*ts*) *sec12-1* yeast mutant (RSY653) with various Bsar1 proteins. Wild-type and mutant *Bsar1* genes were cloned into a site downstream of the *ADH* promoter in *pVT-U*, a yeast expression vector. The *ts sec12-1* mutant yeast was transformed with the *pVT-U* vector (lane 1), the wild-type *Bsar1* gene in a *pVT-U-Bsar1* DNA construct (lane 2), and the point-mutated *Bsar1* genes in a *pVT-U-Bsar1* DNA constructs (lane 3-lane 7). Each transformant was cultured on minimal media plates lacking uracil at 25°C or at 37°C for 5 days

sensitivity (Fig. 6). Since the T51A and T55A mutants showed a severe reduction in GTP-binding activity and GDP-dissociation rates, it may be suggested that the loss of complementation for these mutants reflects conformational changes in Bsar1p itself rather than a loss of the direct interaction between Bsar1p and Sec12p. On the other hand, P54A most likely possesses critical defects in its interaction with Sec12p, since this mutant showed very low GTP-binding and GDP-dissociation compared to the WT protein. Thus, this result provides supporting evidence that P54 is important for interacting with Sec12p. The Pro 54 of Bsar1 is a conserved residue within the effector domain of Sar1 proteins. Crystallographic analysis of the yeast Sec23/24-Sar1 complex revealed that Ser 602 and Glu 605 of Sec23p interact with switch 1 residues of WHP (55-57) in Sar1-GTP [Bi *et al.*, 2002]. Here, we show that the Pro 54 in Bsar1 (corresponding to P57 in yeast sar1p) has a critical role in the interaction with both of these regulators, which is a crucial step in the control of ER-to-Golgi transport by Sar1p.

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