The PHD Domain of Plant PIAS Proteins Mediates Sumoylation of Bromodomain GTE Proteins

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PIAS (protein inhibitor of activated STAT)‡ proteins have generally been accepted to function as transcriptional coregulators (1), but in the last years an emergent role as facilitators of SUMO modification of proteins is becoming clear (2). The small polypeptide SUMO modifies function of proteins involved in a wide range of processes, for instance DNA repair, transcriptional regulation, genome stability, protein stability, and cellular localization of proteins (3). Covalent attachment of SUMO occurs through ε-amino groups of Lys residues, typically found at the consensus sequence ΨKX(E/D) (Ψ, large hydrophobic residue; X, any residue) (4). The process involves the E1-mediated ATP activation of mature SUMO, transfer to catalytic Cys of the E2 conjugating enzyme, and covalent attachment to the target, frequently arbitrated by SUMO E3 ligases, such as PIAS proteins (5).

The presence of a SP-RING (from SIZ/PIAS-RING) domain in PIAS proteins (2), related to the RING finger found in many ubiquitin E3 ligases and able to interact with the E2 enzyme (6, 7), has reinforced the idea that PIAS proteins play a role as SUMO E3 ligases. Other domains may specify target selection or participate in ligase function or different activities. Besides the SP-RING finger, a SAP (from SAF/ACINUS/PIAS) domain, which associates to DNA sequences of matrix attachment regions (MAR) (8), and a non-covalent SUMO interacting motif (SIM) (9) are also distinctive of PIAS proteins.

The complete SUMO pathway has been identified in Arabidopsis (10). This includes the E1 heterodimer AtSAE1/AtSAE2, the E2 AtSCE1, several SUMO proteins (AtSUM), and a unique PIAS protein, AtSIZ1. Intriguingly, besides the characteristic domains, AtSIZ1, as other plant PIAS proteins, also includes a PHD domain. We found that the PHD domain binds AtSCE1 and contributes to the SUMO ligase function, being partially and absolutely required for AtSCE1 and GTE3 sumoylation, respectively. Based on the capacity of AtSCE1 and GTE3 to associate with both the PHD and SP-RING domains, we propose a model of interactions to explain AtSIZ1-mediated sumoylation of GTE3 and ligase function of the PHD domain.

Covalent attachment of small ubiquitin-like modifier (SUMO) to proteins regulates multiple processes in the eukaryotic cell. In numerous cases sumoylation is facilitated by protein inhibitor of activated STAT (PIAS) proteins, characterized by the presence of a SP-RING domain related to the RING finger of many ubiquitin E3 ligases. The importance of SP-RING relies on its capacity to bind the E2 enzyme of the pathway. Additional domains may participate in SUMO ligase function and target selection. We have studied the Arabidopsis SUMO ligase AtSIZ1, belonging to the PIAS family, and describe self-sumoylation and AtSIZ1-mediated sumoylation of the E2 enzyme AtSCE1 and GTE3, a bromodomain protein interacting with AtSIZ1. Modification of GTE3 modulates its capacity to bind acetyl-histone H3 in vitro. Interestingly, AtSIZ1, as other plant PIAS proteins, also includes a PHD domain. We found that the PHD domain binds AtSCE1 and contributes to the SUMO ligase function, being partially and absolutely required for AtSCE1 and GTE3 sumoylation, respectively. Based on the capacity of AtSCE1 and GTE3 to associate with both the PHD and SP-RING domains, we propose a model of interactions to explain AtSIZ1-mediated sumoylation of GTE3 and ligase function of the PHD domain.
PHD-mediated Sumoylation of Bromodomain GTE Proteins

TABLE 1
Proteins produced for this study

| Protein or Mutant | Short name or Accession number |
|-------------------|--------------------------------|
| AtSAE1 (E1)       | E1 At5g50580                   |
| AtSAE2 (E1)       | E1 At2g21470                   |
| AtSUM1 (SUMO1)    | SUMO At4g26840                 |
| AtSCE1 (E2)       | SCE (E2) At5g57970             |
| FLAG-AtSCE1       | FI-SCE                        |
| His<sub>6</sub>-AtSAE1 | His-SCE                      |
| FLAG-AtSAE1 C49S  |                     |
| FLAG-AtSAE1<sup>1–145</sup> (ΔC-ter) |                   |
| FLAG-AtSAE1 K146R/ K147R | |

| AtSIZ1 (E3)       | SIZ At5g60410                  |
| AtSIZ1 SAP mutant | ΔSAP                           |
| AtSIZ1 PHD mutant | ΔPHD                          |
| AtSIZ1 SP-RING mutant | ΔRING                      |
| AtSIZ1 PHD/RING mut. | ΔPHD/RING                  |
| AtSIZ1 SIM mutant | ΔSIM                          |
| AtSIZ1 K100R      | K100R                         |
| AtSIZ1 K488R      | K488R                         |
| AtSIZ1 K100R/K488R| K100/488R                     |
| AtSIZ1<sup>1–36</sup> (ΔC-ter) | SIZΔC                       |
| PHD domain        | PHD                           |
| SP-RING domain    | RING                          |
| SAP domain        | SAP                           |
| His<sub>6</sub>-RanGAP1 | Ran U88155                  |
| FLAG-GTE3         | GTE3 At1g73150                |
| FLAG-GTE5         | GTE5 At1g17790                |
| FLAG-GTE7         | GTE7 At5g65630                |
| FLAG-AtPCNA1      | PCNA At1g07370                |
| FLG-AtPHR1        | PHR1 At4g29610                |

<sup>a</sup> See “Experimental Procedures” for details.
<sup>b</sup> Nucleotide.

Proteins produced for this study

**Proteins and Pulldown Assays**—Proteins (Table 1) were produced in *Escherichia coli* DH5α or BL21 strains as GST or His-tag fusions from the pGEX-6P (GE Healthcare) or pET28 (Novagen) vectors and purified with glutathione-Sepharose 4B (GE Healthcare) or His-Bind Resin (Novagen), respectively. GST fusions were eluted with glutathione or excised from GST by using PreScission Protease (GE Healthcare). For detection, FLAG or His tags were N-terminal-placed, and standard Western blot procedures were applied by using monoclonal anti-FLAG (M2, Sigma) or anti-His (GE Healthcare) antibodies. AtSAE1, AtSAE2, and AtSIZ1 and derived mutants were purified as GST fusions. AtSUM1 was purified as GST fusion or excised from GST. Other GST fusions were excised from GST. For production of C-terminal RanGAP1 we used the pET28RanGAP1-C2 construction (16). In vitro translation in the presence of [35S]Met was performed with the T7 TNT Quick Coupled Transcription/Translation Systems (Promega). For pulldown, matrix-bound GST or GST fusions (0.5–1 μg) were incubated with purified (500 ng) or in vitro translated (one-tenth of the reaction) proteins in 200 μl of buffer 1 (20 mM Tris-HCl, pH 7.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40) and washed with buffer 1 and buffer 1 supplemented with 400 mM NaCl. Samples were boiled in Laemmli buffer and analyzed by SDS-PAGE. For binding to peptides, 2 μg of biotinylated acetylhistone H3 peptide (Upstate Millipore) were incubated with 0.5 μg of protein in buffer 2 (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, 0.25% bovine serum albumin). Proteins were pulled down with streptavidin-coupled magnetic beads (Dynabeads M-280 Streptavidin, Dynal, Invitrogen). Washes and elution were as formerly indicated. Western blot procedures, registration of chemiluminescence with a ChemiDocXRS apparatus (Bio-Rad), or registration of radioactive areas with an InstantImager Electronic Autoradiography apparatus (Packard Instrument Co.) were used for protein detection.

**Sumoylation Assays**—Assays were carried out in 10 μl of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 4 mM MgCl<sub>2</sub>, 1 mM DTT, 10 μM ZnSO<sub>4</sub> for 90 min at 30 °C in the presence of Arabidopsis sumoylation proteins (100 ng of AtSAE1/AtSAE2 mix (E1), 50–500 ng of AtSCE1 (E2), 1 μg of AtSUM1 (SUMO), and 25 ng of AtSIZ1 when required). One-tenth of an in vitro translation reaction was used for sumoylation assays when using AtSIZ1 as a target. In other assays AtSIZ1 or derivatives were used as GST fusions. 50 ng of a purified tagged protein were typically used as the target. Reactions were initiated with 2 mM ATP and usually stopped with SDS and β-mercaptoethanol-containing Laemmli buffer. To discriminate between sumoylation and SUMO loading on AtSCE1, samples treated with 4 μM urea-containing Laemmli buffer, which enables visualization of both SUMO loading and sumoylation, were compared with samples heated in 100 mM DTT-containing Laemmli buffer that, similar to β-mercaptoethanol-containing Laemmli buffer, efficiently eliminates SUMO loading but not sumoylation.

**Yeast Two-hybrid Assays**—Assays and screening were conducted in *Saccharomyces cerevisiae* AH109 on selective medium lacking histidine. pGBK7 and pGA10 plasmids (Clontech) were used for Gal4 DNA binding and activation domain fusions, respectively. We screened a pGAD10-based Arabidopsis inflorescence cDNA library kindly provided by J. L. Richmann (the Meyerowitz laboratory, California Institute of Technology). Two-hybrid mutant constructs of AtSIZ1 were derived from that used for the screening.

**DNA Gel Shift Assays**—A 32P-labeled ClaI-ScaI 300-bp DNA probe corresponding to a MAR sequence near the plastocyanin locus (17) was used for shift assays in 6% acrylamide gels. Reactions were performed in 16 μl of 50 mM Tris-HCl, 50 mM NaCl, 10% glycerol, 5 mM EDTA, 1 mM DTT, containing 10 ng of poly(dl-dC) and 0.5 μg bovine serum albumin with 50 ng of protein for 20 min at room temperature.
PHD-mediated Sumoylation of Bromodomain GTE Proteins

We then proceeded to identify SUMO attachment consensus sites in AtSIZ1. Three consensus sites could be identified with core Lys residues at positions 100, 450, and 488 (Fig. 1A). Lys to Arg mutations revealed that Lys-100 and Lys-488 were critical for modification, and the double mutation K100R/K488R highly impaired AtSIZ1 self-sumoylation (Fig. 1D). Therefore, we have identified two sites for SUMO attachment in AtSIZ1 and emphasized the importance of the SP-RING finger in self-modification.

AtSIZ1 Mediates Covalent Attachment of SUMO to E2—In the second step of sumoylation SUMO is transferred from E1 to the catalytic Cys of E2 (C94 in AtSCE1) to form an E2-SUMO thioester. Besides this process of SUMO loading, covalent attachment of SUMO to E2 (sumoylation) has also been described (19, 20). We detected AtSIZ1-dependent modification of AtSCE1 (Fig. 2A). Because samples were treated with β-mercaptoethanol-containing Laemmli buffer that eliminates SUMO loading, it should correspond to sumoylation. Moreover, a C94S mutant (impaired in SUMO loading) was also modified when adding in trans non-tagged wild type (WT) AtSCE1 (Fig. 2A), indicating that a modification different to SUMO loading occurs. To better discriminate between SUMO loading and sumoylation, we compared samples treated with urea, which enables visualization of both SUMO loading plus sumoylation, with samples treated with DTT that, similar to β-mercaptoethanol, efficiently eliminates SUMO loading but not sumoylation. This approach let us to confirm AtSIZ1-mediated sumoylation of AtSCE1 (Fig. 2B), it is worth noting that urea treatment also permits visualization of protein dimers not related to SUMO loading or sumoylation.

A specific Lys at the C terminus has been previously described as the site for sumoylation of yeast and human E2 (19, 20). This Lys corresponds to Lys-154 in AtSCE1. We tried to eliminate Lys-146, Lys-147, Lys-150, and Lys-154 by deleting the 15 C-terminal residues of AtSCE1 (from Lys-146), but this deletion rendered inactive AtSCE1 (not shown). Otherwise, Lys to Arg mutation of these residues did not impair SUMO attachment (not shown). Thus, besides SUMO loading, AtSCE1 is susceptible of SUMO attachment mediated by AtSIZ1.

The AtSIZ1 PHD Domain Contributes to AtSCE1 Sumoylation—Because AtSCE1 modification depends on AtSIZ1, we investigated the involvement of its different domains in this process. Different AtSIZ1 mutants (Fig. 1A) were assayed for their ability to facilitate SUMO attachment to AtSCE1. Our results indicated that SP-RING disruption greatly, but not completely, impaired AtSCE1 sumoylation (Fig. 2C). Because SP-RING and PHD domains may display common structural features, we analyzed the involvement of PHD in this process. No apparent defects were observed with a PHD mutant; however, low sumoylation detected in the absence of the SP-RING finger was completely abolished when using a double PHD/SP-RING mutant (Fig. 2D). Then, although the SP-RING finger seems to be essential for efficient SUMO modification of AtSCE1, the PHD domain also contributes to this function.

AtSCE1 Binds to the AtSIZ1 PHD Domain—Because the relevance of SP-RING has been based on its ability to interact with E2 (6, 7), we proceeded to a detailed analysis of the AtSIZ1-

RESULTS

SUMO Consensus Sites and the SP-RING Domain Facilitate AtSIZ1 Self-sumoylation—Self-sumoylation is characteristic of many PIAS proteins (18). To investigate ligase function of AtSIZ1, we first decided to analyze this process. Sumoylation assays with purified Arabidopsis proteins, and in vitro translated WT SIZ or derived mutants were assayed for sumoylation. By, sumoylation assay of WT SIZ with different combinations of E1, AtSCE1 (SCE), SUMO, and GST-SUMO. C. WT and different SIZ mutants were assayed for self-sumoylation (+ATP). D. WT SIZ and mutants of core Lys at sumoylation consensus sites were assayed for self-sumoylation (+ATP).

FIGURE 1. AtSIZ1 self-sumoylation. A, schematic representation of WT AtSIZ1 (SIZ) and derived mutants used in this study. B–D, 35S-labeled in vitro translated WT SIZ or derived mutants were assayed for sumoylation. E1, sumoylation assay of WT SIZ with different combinations of E1, AtSCE1 (SCE), SUMO, and GST-SUMO. C. WT and different SIZ mutants were assayed for self-sumoylation (+ATP). D. WT SIZ and mutants of core Lys at sumoylation consensus sites were assayed for self-sumoylation (+ATP).
PHD-mediated Sumoylation of Bromodomain GTE Proteins

FIGURE 2. AtSIZ1 mediates AtSCE1 sumoylation through PHD and SP-RING domains. A, FLAG tagged WT AtSCE1 (Fl-SCE) or the AtSCE1 C94S mutant (Fl-C94S) were assayed for SUMO modification (+ATP) with different combinations of SUMO, AtSIZ1 (SIZ), and non-tagged WT AtSCE1 (SCE) added in trans. B, SUMO modification of Fl-SCE or Fl-C94S was analyzed as in A, but samples were treated with DTT (reducing conditions), which eliminates SUMO loading but not sumoylation, or urea (non-reducing conditions), that enables visualization of both SUMO loading and sumoylation (see “Results”). L and S refer to the protein-SUMO complexes as SUMO loading or sumoylation, respectively. Under non-reducing conditions some protein dimers were observed. C and D, Fl-SCE (−) was assayed for SUMO modification in the absence (−SIZ) or the presence of WT AtSIZ1 (+SIZ) or derived mutants (see Fig. 1A). Proteins were revealed with anti-FLAG antibodies.

AtSCE1 interaction to clarify how the PHD domain participates in sumoylation. We carried out two-hybrid assays (Fig. 3A) with AtSCE1 and several AtSIZ1 constructions derived from that used in our two-hybrid screening (see below). Growth of yeast harboring a PHD mutant construction of AtSIZ1 was similar to that of yeast carrying the WT construction. On the other hand, SP-RING disruption significantly reduced the interaction, which eliminates growth completely by using a double PHD/SP-RING mutant construct. Pulldown assays using matrix-bound GST-AtSCE1 protein and in vitro translated AtSIZ1 mutants or matrix-bound GST fusions of different AtSIZ1 domains and purified FLAG-AtSCE1 confirmed the interactions identified by yeast two-hybrid (Figs. 3, B and C).

Because the C terminus of RanGAP1 is a substrate of E2 independent of SUMO ligases (16) but AtSIZ1 binds AtSCE1, we speculated that AtSIZ1 may have a negative effect on AtSCE1-mediated sumoylation of RanGAP1. In fact, interference of AtSIZ1 and derived mutants with RanGAP1 sumoylation was proportional to the level of interaction with AtSCE1 (Fig. 3D). Besides the ability of AtSCE1 to interact with SP-RING, we have also revealed an interaction with the PHD domain, which may account for a SUMO ligase function.

A Two-hybrid Screening Identifies GTE3 as a Partner of AtSIZ1—We subsequently investigated the AtSIZ1-mediated attachment of SUMO to AtSCE1 and to itself. To further investigate the ligase function of AtSIZ1, we decided to perform a two-hybrid screening to identify AtSIZ1 targets not involved in the SUMO pathway. Because full-length AtSIZ1 fused to Gal4 DNA binding domain transactivated two-hybrid reporters in the absence of a partner interaction, we used a truncated protein (amino acids 1–536, SIZAC), encompassing the SAP, PHD, and SP-RING domains as bait, which retained the ability to interact with AtSCE1 (Fig. 3A). Among the positive clones isolated, we identified AtSCE1 and GTE3 (At1g73150) (Fig. 4A), belonging to the BET (bromodomain and extra terminal domain) family of proteins (21). Pulldown experiments demonstrated a direct and specific interaction (Fig. 4B). Importantly, GTE3 showed a pattern of interaction very similar to that observed for AtSCE1, i.e. SP-RING mediated major interaction, but the PHD domain also contributed (Fig. 4A).

Twelve different GTE (Global transcription factor group E) proteins have been described in Arabidopsis (ChromDB Chromatin Data base). We wondered about the specificity of AtSIZ1 interaction with other GTE members. We used the two-hybrid approach to test interaction with GTE5 (At1g17790), a protein displaying high homology (80% identity) with GTE3, and GTE7 (At5g65630) and GTE10 (At5g63330), displaying only 43% identity with GTE3. The analysis revealed interaction of AtSIZ1 with GTE5 but not with GTE7 or GTE10 (Fig. 4C). Then, our two-hybrid screening, validated by the identification of AtSCE1 among the positive clones, has also revealed a member of the GTE family as a partner of AtSIZ1.

AtSIZ1 Acts as a SUMO Ligase of GTE3—We subsequently addressed whether the AtSIZ1-GTE3 interaction mediated GTE3 sumoylation. Interestingly, GTE3 was susceptible of SUMO attachment, and this depended on AtSIZ1 (Fig. 5A). To check for target specificity we assayed other proteins. Proliferating cell nuclear antigen (PCNA) has previously been described as a SUMO substrate in yeast, and SIZ/PIAS seems to stimulate its modification (22); however, we found that AtPCNA1 is not modified in the presence of AtSIZ1 (Fig. 5B). On the other hand, sumoylation of the Arabidopsis phosphate-related MYB transcription factor AtPHR1 has also been previously demonstrated (12). Then, under our assay conditions it was modified, although not as efficiently as GTE3 (Fig. 5B). Because GTE5 but not GTE7 interacted with AtSIZ1, we also proved AtSIZ1-dependent sumoylation of these proteins. Accordingly with the interactions described, GTE5 but not GTE7 was susceptible of sumoylation (Fig. 5C).

Because bromodomains have been demonstrated to bind acetylated histone tails (23), we decided to investigate if GTE3 bound to acetylhistone H3. As shown in Fig. 5D, GTE3 was retained by a peptide corresponding to acetylhistone H3 N-terminal tail (acH3), whereas AtSCE1, used as a control, was not. Then we wondered about the consequences of sumoylation in binding to acH3. To investigate this, after sumoylating GTE3, half of the reaction was used in pulldown experiments with acH3 and the remaining fraction was monitored as the input. Quantification of protein bands indicated that in contrast to 77% of non-modified GTE3, only 12% of sumoylated GTE3 was retained by the acH3 peptide (Figs. 5, E and F). In sum, certain members of the
GTE family are specific targets of the SUMO ligase function of AtSIZ1, and sumoylation would modulate interaction of these proteins with other partners such as acetylated histone tails.

AtSIZ1-mediated Sumoylation of GTE3 Requires the PHD—
We then proceeded to study the implication of the different AtSIZ1 domains in SUMO transfer to GTE3. Surprisingly, the PHD mutant was as inefficient as that lacking the SP-RING domain in promoting GTE3 modification (Fig. 6A). Even more, a double PHD/SP-RING mutant molecule did not behave differently to single mutants (Fig. 6A). Similar results were obtained with GTE5 (not shown).

To ensure that mutations affecting AtSIZ1-mediated SUMO transfer did not compromise other AtSIZ1 activities, we inves-
tigated the capacity of mutant proteins to bind a DNA MAR sequence. For that, we used a MAR sequence associated to the plastocyanin locus (17). This function typically depends on the SAP domain, and accordingly, only the SAP mutant was affected (Fig. 6B).

Because PHD is used to mediate protein-protein interaction and may facilitate dimerization, which might be important for E3 function of AtSIZ1 on certain proteins, we analyzed AtSIZ1 dimer formation in two-hybrid assays, but we did not detected interaction (not shown). Hence, the AtSIZ1 PHD mutant is highly impaired in SUMO transfer to GTE3 as the one lacking the SP-RING, but these mutations do not affect another PIAS function, the binding to a MAR sequence.

In the Absence of the PHD Domain, AtSCE1 and GTE3 Compete for Binding to AtSIZ1—We have revealed a similar pattern of interaction of AtSCE1 and GTE3 with AtSIZ1 domains. This may lead to competition for binding and be reflected on ligase capacity. In the absence of the PHD domain, no AtSIZ1-mediated modification of GTE3 is detected. As PHD seems to constitute an alternative to the SP-RING for AtSCE1 and GTE3 association, this may anticipate in its absence, E2 (AtSCE1) and target (GTE3) compete for SP-RING binding and that both proteins mutually exclude each other. Then, we decided to investigate competition. Because GTE3 sumoylation obviously requires AtSCE1 (Fig. 5A), it is difficult to assess a competition effect of AtSCE1 on GTE3 sumoylation. By contrast, by using the PHD mutant, inactive in GTE3 sumoylation (Fig. 6A) but efficient in AtSCE1 modification (Figs. 2, C and D), we can measure AtSCE1 sumoylation in the presence of GTE3 as a reflection of competition of both proteins for binding to AtSIZ1. Results indicated that a 5-fold increase in GTE3 concentration abrogated AtSCE1 modification, whereas a similar concentration of other proteins such as AtPHR1 or AtPCNA1 had no effect (Fig. 7A). Moreover, competition of AtSCE1 and GTE3 for binding to the SP-RING domain was corroborated in pulldown experiments (Fig. 7B). Therefore, these results provide evidence that when mutating the PHD domain, GTE3 and AtSCE1 may compete for binding to AtSIZ1.

DISCUSSION

AtSIZ1 has been implicated in phosphate starvation responses, salicylic acid-dependent pathogen defense, flowering, basal thermotolerance, and cold tolerance in Arabidopsis (24), but SUMO ligase function on specific targets has not been ascribed to particular AtSIZ1 domains. In this study we describe AtSIZ1 self-sumoylation and AtSIZ1-mediated sumoylation of AtSCE1 and GTE3 and attribute a SUMO ligase function in the modification of these proteins to the PHD domain.

We have discriminated between SUMO loading and covalent attachment of SUMO to AtSCE1 and found that the last is dependent on AtSIZ1. Sumoylation of PIAS and E2 proteins has previously been observed (18–20). However, modification of E2 has not been established as PIAS-dependent. Self-sumoyla-
AtSCE1 (His-SCE) was assayed for SUMO modification (Fig. 2). Independent sumoylation of different targets (Fig. 2) mutations impairing self-modification do not affect AtSIZ1-deactivated sumoylation in yeast and human E2 enzymes (19, 20). Lys to Arg mutation of putative target Lys residues did not disturb AtSCE1 sumoylation, but deletion of the 15 C-terminal amino acids inactivated the enzyme, suggesting that the C terminus has relevant functions. The importance of the C-terminal region in E2 activity has been previously suggested (27).

Several lines of evidence derived from our interaction and sumoylation analysis support GTE3 as an AtSIZ1 target; (i) GTE3 has been isolated from a two-hybrid screening with AtSIZ1, (ii) pulldown experiments have shown direct and specific interaction of both proteins, (iii) the interaction surfaces in AtSIZ1 have been mapped to the PHD and SP-RING domains, (iv) GTE3 sumoylation depends on AtSIZ1, (v) mutation of specific functional domains of AtSIZ1 impairs GTE3 sumoylation, and finally, (vi) AtSIZ1 shows significant specificity for GTE3 sumoylation. Specificity is also appreciable within the GTE family as GTE3 and GTE5 behave as AtSIZ1 targets, whereas GTE7 and GTE10 do not interact with AtSIZ1, and consequently, this does not mediate GTE7 sumoylation. Because GTE5 displays a high homology with GTE3, specific domains in these proteins, not present in other GTEs, should account for AtSIZ1 interaction. Under our assay conditions we also get sumoylation of AtPHR1, a previously described AtSIZ1 target (12), although it is not as efficient as with GTE3. By contrast, AtPCNA1 is not modified in our assays. PCNA has been described as a Siz/PIAS target in yeast, but sumoylation of PCNA has been established as Siz1- and not Siz2-dependent (22). There is no evidence of PCNA sumoylation in Arabidopsis to date; however, we cannot rule out the possibility that it would be SUMO-modified under the control of an E3 other than AtSIZ1. Interestingly, Lys-127 has been previously determined as the lysine for sumoylation of yeast PCNA in vitro (28), and this residue is not conserved in AtPCNA1.

The BET family in plants (GTE) has been poorly investigated. GTE1 (also called IMB1) has been implicated in germination (29), whereas GTE6, a distant-related GTE member, has been involved in leaf morphology (30). In contrast, the transcriptional role of vertebrate BET proteins (Brd) is well documented (31), and binding of Brd to acetylated histones has been shown (32). Similarly we have proved GTE3 interaction with acetylated histone H3. Even more, our results indicate that sumoylation weakens binding of GTE3 to acetylated histone tails. A role of SUMO in modulating interaction or affinity has been previously reported. For instance, thymine DNA glycosylase (TDG) removes mismatched T and U, creating abasic sites in the DNA to which TDG remains tightly bound. SUMO modification reduces binding capacity and lets recycling of thymine DNA glycosylase for a next reparation event (33).

Recognition of the consensus motif ΨKX(E/D) by the E2 enzyme may participate in initial selection of proteins as SUMO targets (4, 34). Subsequently, PIAS proteins would select and specify proteins for SUMO transfer. As E3 ligases, they have adaptor functions that bring together SUMO-loaded E2 and targets and facilitate appropriate interaction for sumoylation. In this process, the SP-RING finger, as an E2 interacting module, has a relevant role. Besides this module, exclusive domains in the different PIAS proteins should account for target selection and specificity. This has recently been emphasized for Siz proteins in yeast (22). Both PHD and SP-RING structures resemble that of RING fingers. Interestingly, PHD domains have been implicated in ubiquitin ligase activity (35, 36). However, this matter remains controversial, as this has been refuted, arguing that conclusions were derived from misinterpretation of RING domains as PHD (11). SMART (37) and PFAM (38)
PHD-mediated Sumoylation of Bromodomain GTE Proteins

**A** WT AtSIZ1 | mutant AtSIZ1

![Diagram](image)

**B**

| Protein | SAP | PHD | SP-RING | Length (aa) | PHD |
|---------|-----|-----|---------|-------------|-----|
| P. vivax |  | 🟢 | 🟢 | 1060 | 181-233 |
| M. trunc | 🟢 | 🟢 | 🟢 | 882 | 118-170 |
| O. sativa | 🟢 | 🟢 | 🟢 | 880 | 124-177 |
| A. thali | 🟢 | 🟢 | 🟢 | 873 | 114-166 |
| PIASxB | 🟢 | 🟢 | 🟢 | 621 |  |

**C**

![Sequence Alignment](image)

**FIGURE 8. A role of the PHD domain of plant PIAS proteins in sumoylating GTE proteins.** A. WT AtSIZ1 (left) in the absence of GTE3 (GTE, upper part) may fix AtSCE1 (SCE) to both PHD and SP-RING, and SUMO (S) transfer would occur within a single SCE molecule or between adjacent molecules. In the presence of GTE3 (lower part), each protein fix to a different module (the figure shows only one of two possibilities), and both proteins may be sumoylated. When PHD or SP-RING is mutated (right), SCE may still fix AtSIZ1 (upper part) and be sumoylated, but in the presence of GTE (lower part) both proteins should compete for fixation, excluding simultaneous binding and consequently avoiding GTE sumoylation. The dashed arrow indicates that AtSCE1 self-sumoylation would still occur but might be compromised. SUMO loading and sumoylation are indicated by arrowheads and ←, respectively. B, schematic representation of Plasmodium (P. vivax) and plant PIAS proteins from Medicago truncatula, O. sativa, and Arabidopsis (A. thali). PIASxB from mouse is also included as a PIAS protein lacking PHD. Length of proteins is given in amino acids (aa). The position of PHD is also indicated. Accession numbers in the same order are AS52L3, Q1RWA4, A3AM50, Q680Q4, Q8C5D8. C, sequence alignment of PHD domains of proteins in B. Conserved residues are in shadow. For comparison, the SP-RING domain of Arabidopsis has also been included (amino acids 362–406). Black and white arrowheads indicate residues that define PHD (C4HC3) and SP-RING (C2HC3) domains, respectively.

The analysis of the AtSIZ1 amino acid sequence reveals the presence of both PHD and SP-RING domains. Here we have shown that besides SP-RING, the PHD domain is able to bind the E2 enzyme and contribute to SUMO ligase function of a PIAS protein. While this manuscript was under revision Ivanov et al. (39) reported that the PHD domain of the KAP1 corepressor functions as an E3 ligase for intramolecular sumoylation in animal cells. Then, our results together with those of Ivanov et al. (39) are the first evidence of a SUMO ligase function coupled to PHD. Moreover, the role of a PHD domain in a PIAS protein had not been investigated hitherto. Other SUMO ligases as Pc2 (40) and RanBP2 (41), which share no significant homology with PIAS proteins, are also able to mediate E2 interaction and SUMO transfer, supporting the idea that additional domains other than the SP-RING finger may mediate sumoylation.

Although the PHD modestly contributes to AtSCE1 sumoylation, it is absolutely required for GTE3 modification. Our results indicate that in the absence of the PHD, these proteins compete for binding to AtSIZ1 and mutually exclude each other. Because the PHD domain contributes to AtSIZ1 interaction with AtSCE1 and GTE3, we propose that when GTE3 occupies one of the PHD or SP-RING domains, AtSCE1 may fix to the open one to mediate sumoylation (Fig. 8A). Alternatively, both the PHD and SP-RING domains might together comprise a unique binding site for AtSCE1 and GTE3. However, because the AtSIZ1 PHD mutant behaves in a quite different manner in sumoylating AtSCE1 versus GTE3 and AtSCE1 independently binds PHD and SP-RING, we prefer to favor the existence of two binding modules. Thus, sumoylation of AtSCE1 requires only one working module, whereas sumoylation of GTE3 requires both to allow simultaneous binding of AtSCE1 and GTE3. Such a peculiarity of having an alternative module for E2 may add functional versatility to a PIAS protein. Then, association of other proteins with one of the two E2 interacting motives may condition binding of E2 and serve as a way to modulate function. Otherwise it may account for an extended number of targets.

Interestingly, a data base search indicates that besides Plasmodium, proteins with SAP and SP-RING motives (hallmarks of PIAS proteins), also harboring a PHD domain, are found in Arabidopsis, Oryza sativa, and Medicago truncatula (Figs. 8, B and C). Actually, PIAS proteins lacking PHD seem to be absent from plants. These observations raise the question of the association of such architecture especially with plant PIAS proteins.

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