Repression of the Transactivating Capacity of the Oncoprotein PLAG1 by SUMOylation*

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Human pleomorphic adenoma gene 1 (PLAG1), a developmentally regulated proto-oncogene, is consistently rearranged and overexpressed in pleomorphic salivary gland adenomas and lipoblastomas with 8q12 translocations. Together with PLAGL1 and PLAGL2, PLAG1 belongs to a subfamily of C2H2 zinc finger transcription factors that activate transcription through binding to the bipartite consensus sequence GRGGC(N)₆₋₈GGG. Ectopic expression of PLAG1 deregulates target genes and presumably results in uncontrolled cell proliferation. To gain insight into molecular mechanisms regulating PLAG1 transcriptional capacity, we searched for interaction partners using the yeast two-hybrid system and confirmed these by glutathione S-transferase pull-down. Ubiquitin-conjugating enzyme 9 (UBC9) and protein inhibitor of activated STAT (PIAS) proteins were first identified as genuine interacting partners of mouse PlagL2. Because UBC9 and PIAS are components of the small ubiquitin-related modifier (SUMO) modification pathway, we hypothesized that PLAG proteins could be SUMOylated. Here, we report results obtained for founding family member PLAG1. Its endogenous SUMOylation was demonstrated, and SUMOylation of PLAG1 was further investigated in cells co-transfected with PLAG1 and SUMO-I DNA or a SUMO-I mutant form and similarly examined in the presence or absence of DNA encoding the various PIAS proteins. Using anti-PLAG1 antibodies, we discovered single and double SUMO-1-modified forms of PLAG1. By mutating predicted SUMO consensus sites, we defined two important target lysines for SUMOylation in PLAG1, Lys-244 and Lys-263. Moreover, mutation of both SUMO consensus sequences, resulting in inhibition of SUMOylation, led to a significant increase of the transactivation capacity of PLAG1. Nuclear distribution of PLAG1 was not measurably influenced. Our results suggest a direct repression of the transactivating capacity of the oncoprotein PLAG1 by SUMOylation.

Pleomorphic adenoma gene 1 (PLAG1) is a proto-oncogene on human chromosome 8q12, the oncogenic activation of which is a crucial event in the formation of pleomorphic adenomas of the salivary glands (1) as well as lipoblastomas (1, 2). PLAG1 encodes a zinc finger transcription factor, and the PLAG1 (PLAG-like 1) and PLAG2 (PLAG-like 2) genes (3) constitute two structurally related family members (1). The main mechanism of oncogenic activation involves recurrent chromosome translocations leading to promoter substitution between PLAG1, a gene primarily expressed in various tissues during fetal development, and more broadly and constitutively expressed genes. Chromosome breakpoints invariably occur in the 5′-non-coding region of PLAG1, causing an exchange of regulatory control elements without structurally affecting the coding sequences of the PLAG1 gene. This process, also known as promoter swapping, leads to ectopic expression of PLAG1 in the tumor cells. It is assumed that such abnormal PLAG1 expression causes a deregulation of expression of PLAG1 target genes and leads to tumor development in these tissues (1, 4–6). Furthermore, ectopic PLAG1 expression has also been found in tumors without 8q12 translocations, such as pleomorphic salivary gland adenomas with chromosome 12q15 translocations or with a normal karyotype, in leiomyomas of the uterus, and in leiomyosarcomas (5). The involvement of the PLAG1 proto-oncogene in the development of various tumor types emphasizes its more general importance in tumorigenesis.

The oncogenic potential of PLAG1 has been established by various experimental approaches (7). Overexpression of PLAG1 in NIH-3T3 cells leads to cell proliferation in serum-deprived (1%) medium, suggesting that PLAG1 partially abrogates the serum requirement for the growth of these cells. These PLAG1-overexpressing NIH-3T3 cells also displayed the typical transformation hallmarks, i.e. the cells no longer possess cell-cell contact inhibition, show anchorage-independent growth and, upon injection into nude mice, develop into tumors. Structurally, the PLAG1 transcription factor contains seven canonical C₂H₂ zinc fingers and a serine-rich C terminus with transactivation capacity (8). PLAG1 specifically recognizes a bipartite DNA-binding consensus sequence consisting of a core sequence, GRGGC, and a G-cluster, GGG, which are separated by about 6–8 nucleotides (8). In recent microarray analyses, genes were identified that are consistently induced or repressed by PLAG1, and these were classified into various functional categories. Among the classes of up-regulated PLAG1 targets, the one of growth factors was the largest and

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§ The abbreviations used are: PLAG1, pleomorphic adenoma gene 1; PLAGL1, pleomorphic adenoma gene-like 1; PLAGL2, pleomorphic adenoma gene-like 2; GST, glutathione S-transferase; PIAS, protein inhibitor of activated STAT; STAT, signal transducer and activator of transcription; UBC9, ubiquitin-conjugating enzyme 9; SUMO, small ubiquitin-related modifier; SSPI, SUMO-specific protease 3; PML, promyelocytic leukemia protein; RanBP2, Ran-binding protein 2; LEF1, lymphoid-enhancing factor 1; GS, glutathione-S-Sepharose; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; GFP, green fluorescent protein; m, mouse; SENS, Sentrin/SUMO-specific protease.
included insulin-like growth factor II, cytokine-like factor-1 (CLF-1), bone-derived growth factor (BPGF-1), choriogonadotropin β chain, vascular endothelial growth factor, and placental growth factor. From in silico evaluation of their promoter regions, it appeared that a large proportion of them harbor several copies of the specific bipartite DNA-binding consensus sequence, suggesting that they constitute direct PLAG1 targets. The in silico studies, furthermore, indicated that PLAG1-down-regulated genes are likely to be regulated indirectly by PLAG1 since the typical PLAG1 DNA-binding consensus sequences were not present in their promoter regions (9).

At present, little information is available on the mechanism(s) by which the PLAG1 transcription factor regulates expression of its target genes. Most transcription factors act as part of a complex composed of several polypeptides, each with a distinct function. This, for instance, has already been described for PLAG1, which can function as a transcriptional co-activator or repressor for hormone-dependent activity of nuclear receptors when acting together with other proteins (10). Here, we report about experiments that were designed to identify and characterize PLAG1 interaction partners, with the objective to gain more insight in the protein environment that allows PLAG1 to modulate gene transcription and to reveal the mechanism of regulation of this transactivation capacity. Therefore, yeast two-hybrid screenings were performed, candidate interaction partners were validated by GST pull-down, and selected candidates were functionally evaluated with respect to the transactivating potential of PLAG1.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis—Hemagglutinin-tagged pcDNA3-PLAG1 (8) with single amino acid substitutions K244R, K363R, or K363R, as well as the double mutant K244R/K363R, were constructed using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Mutations were confirmed by nucleic acid sequencing, and such PLAG1-mutated DNA was subcloned into DraIII/Xhol-digested hemagglutinin-tagged pCAGGS-PLAG1 to achieve higher levels of expression (8). Full-length PLAG1 from pcDNA3-PLAG1 was subcloned as an MscI/XhoI DNA fragment into the pEGFP-C3 vector (Clontech), digested with SacI and SalI, and subcloned as a BstXI/Xhol DNA fragment into the pHL vector (Invitrogen), digested with SalI/Xhol. The K244R, K363R, and K244R/K363R mutant forms of these constructs were also generated using the QuikChange site-directed mutagenesis kit.

**Yeast Two-hybrid Analysis—** The DNA fragment of mouse Plagl2, coding for amino acids 1–382, was cloned in the EcoRI/Sall sites of the pBD-Gal4 (Clontech), digested with SacI and SalI, and subcloned as a BstXI/Xhol DNA fragment into the pHL vector (Invitrogen), digested with SalI/Xhol. The K244R, K363R, and K244R/K363R mutant forms of these constructs were also generated using the QuikChange site-directed mutagenesis kit.

**Western Blot Analysis—** Cell extracts from COS7 cells (ATCC CRL1651) were prepared 48 h after transfection in SDS sample buffer (5% SDS, 0.15 M Tris HCl, 0.1% SDS, 0.2% β-mercaptoethanol, 0.15% NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.1% azide), containing 20 μg/ml of PLAG1 antibody (1:3000 diluted) (Bender MedSystems), and 20 μM of HEPES (pH 7.9). After a 15-min incubation at 4 °C, 12.5 μl of Nonidet P-40 10% was added, and the nuclei were spun down at 13,000 rpm. The nuclei were resuspended in a buffer containing 1 mM dithiothreitol, 1 mM EDTA, 10 μg/ml of anti-PLAG1 antibody (1:3000 diluted), and 10 μg/ml of Bovine Serum Albumin (Roche Applied Science) (12). Samples containing equal protein amounts were heated at 95 °C for 5 min, size-fractionated by electrophoresis in 10% polyacrylamide gels, and transferred to nitrocellulose membranes (PROTRAN nitrocellulose transfer membrane, Schleicher & Schuell BioScience). PLAG1 was detected with a rabbit polyclonal anti-PLAG1 antibody (1:3000 diluted), directed against the N-terminal part of PLAG1 (13), followed by an incubation with horseradish peroxidase-conjugated swine anti-rabbit IgG as secondary antibody (PROSAN; DAKO; 1:1000 diluted). Immunoreactive proteins were visualized using Western Lightning chemiluminescence reagent Plus (PerkinElmer Life Sciences) according to the suppliers’ protocols.

**Cellulose Pull-down—** COS7 cells, grown in 6-well plates, were washed twice in phosphate-buffered saline, collected, and spun down. The cell pellet was resuspended in 200 μl of buffer containing 1 mM dithiothreitol, 0.1 mM EDTA, 10 mM KCl, 0.1 mM EGTA and 10 mM HEPES (pH 7.9). After a 15-min incubation at 4 °C, 12.5 μl of Nonidet P-40 10% was added, and the nuclei were spun down at 13,000 rpm. The nuclei were resuspended in a buffer containing 1 mM dithiothreitol, 1 mM EDTA, 0.4 mM NaCl, 1 mM EGTA, and 20 mM HEPES (pH 7.9). Resuspension by vortexing, followed by an incubation step of 10 min at 4 °C, was repeated four times (40 min in total). The samples were frozen quickly in liquid nitrogen and defrozen at 37 °C twice. The nuclear debris was separated from the nuclear lysate by centrifugation in a microcentrifuge.

**Mamalian Pull-down—** COS7 cells, grown in 6-well plates, were collected in microcentrifuge tubes, 48 h after transfection, and a nuclear extraction was performed according to the protocol described above. An equivalent amount of GS beads was used per well. The beads were washed twice in phosphate-buffered saline and twice in immunoprecipitation lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) supplemented with 1 mM NaF (1:100), 0.5 mM NaVO4 (1:500), and complete protease inhibitor mixture (Roche Applied Science). The beads were then added either to the nuclear extract or, as a control, to the cytoplasmic extract. After incubation overnight at 4 °C on a rotator, the beads were washed four times in immunoprecipitation lysis buffer. The samples were analyzed on SDS-PAGE according to the protocol described above. The pulled-down proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with the following antibodies: mouse anti-PLAG1 antibody (1:3000 diluted), whereas SUMOylation of PLAG1 by SUMO-1-Myc or by endogenous SUMO-1 was detected using mouse monoclonal anti-SUMO-1 antibodies (Santa Cruz Biotechnology; 1:1000 diluted). Blots were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:1000 diluted). Specific bands were visualized using ECL (Amersham Bioscience) according to the supplier’s protocol. Membranes were further stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 50 mM Tris-HCl (pH 6.8)). Blots were subsequently reprobed, first with rabbit polyclonal anti-PLAG1 antibody (1:3000 diluted), directed against the N-terminal part of PLAG1 (13), and secondly with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:1000 diluted). Specific bands were visualized using ECL (Amersham Bioscience) according to the supplier’s protocol.

**Fluorescence Microscopy—** COS7 cells were used to examine the expression and subcellular localization of the PLAG1-GFP fusion proteins. Cells were grown to 70–80% confluence on coverslips in 24-well
Fig. 1. The PLAG proteins interact with members of the PIAS protein family. A, schematic representation of protein domains in the PLAG protein family members. At the top of the figure, a schematic overview of the domain structure of the three different PLAG protein family members is shown. In the lower part, the construction of the bait, used for the yeast two-hybrid experiments, is depicted. As indicated in the figure, the first 382-amino acid (AA) fragment of mPlagl2 was expressed in-frame with the yeast Gal4 DNA-binding domain (DBD). Note that this part of mPlagl2 is 98.7% identical to the corresponding part of its human (h) ortholog. B, interaction between the members of the PLAG protein family and the members of the PIAS protein family in a GST pull-down assay. An autoradiograph of 35S-labeled PLAG1, PLAGL1, and Plagl2, recovered after interaction with GST alone, GST-PIASx0, GST-PIASx1, GST-PIAS3, and GST-PIASy, as indicated, is shown. In all cases, 10% of the input amount of 35S-labeled protein, used in the GST pull-down assay, is shown in the input lane.

plates. 42 h after transfection, the cells were fixed for 10 min at room temperature in 4% formaldehyde (diluted in phosphate-buffered saline). Three subsequent wash steps were performed in phosphate-buffered saline. The slides were then mounted in Vectashield (Vector Laboratories, Inc.) supplemented with 1/1000 4',6-diamidino-2-phenylindole for analysis under a fluorescence microscope.

Transcription Reporter Assays—The human fetal kidney epithelial cell line HEK 293 (ATCC CRL 1573) was cultured according to the supplier's protocols. Cells (grown in 24-well plates) were co-transfected in duplicate with 250 ng of the WT3,TKlux reporter plasmid DNA (5) and 100 ng of the various hemagglutinin-tagged PLAG1-encoding DNA constructs, namely PLAG1 DNA (encoding residues 1–500 and subcloned in EcoRI/XhoI-digested pCAGGS) or DNA of the various PLAG1 mutants (i.e. K244R, K263R, or K244R/K263R in pCAGGS), in combination with 100 ng of DNA encoding small ubiquitin-related modifier (SUMO)-Myc, SUMO-Myc-mutant (SUMO-1 in pSG5; Myc-tagged, 2 C-terminal glycines deleted in the case of the mutant form), or SUMO-specific protease 3 (SSP3). The total DNA amount for transfection was kept the same in each sample (500 ng) by normalizing with empty vector. The transfections were each performed using 1.5 μl of FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer's protocol. Cells were harvested 42 h after transfection, and luciferase reporter enzyme activity was measured using a Wallac Victor 1420 Multilabel Counter (PerkinElmer Life Sciences).

RESULTS

Search for Interaction Partners of PLAG Proteins—To address the question as to whether PLAG-modulated gene transcription involves interacting proteins, we started a search for these. Therefore, we performed a pilot yeast two-hybrid screening assay using an already available mouse Plagl2 bait construct, designated B1–382 mPlagl2. B1–382 mPlagl2 encodes amino acid residues 1–382 representing the zinc finger domain region and the N-terminal part of the serine-rich transactivation domain (Fig. 1A). The construct was generated by PCR starting from a double-stranded mPlagl2 cDNA cloned in the pCg-N vector, and its usefulness (the absence of intrinsic transactivation capacity) was first established. Interactions between B1–382 mPlagl2 and several members of the PIAS protein family as well as ubiquitin-conjugating enzyme 9 (UBC9) were observed. By GST pull-down experiments (Fig. 1B), the interaction between full-length mPlagl2 and those PIAS proteins was confirmed and, subsequently, similar results were obtained with the human PLAG1 and PLAGL1 proteins (Fig. 1B). It should be noted that in the case of 35S-labeled PLAG1, the upper band corresponds to the full-length 35S-PLAG1, whereas the lower band corresponds to a smaller form, due to translation starting at an alternative initiation site (ATG). Collectively, these results clearly establish the interaction between the various PLAG and PIAS proteins. This observation as well as the observed interaction between Plagl2 and UBC9 indicate that the PLAG proteins could be subjected to post-translational modification via covalent SUMOylation since UBC9 and PIAS proteins act as E2 and E3 ligases, respectively, in the SUMO modification pathway. To address this question, SUMOylation studies were performed, and these all focused on the PLAG1 protein, the founding member of the family.

PLAG1 Is SUMOylated, and This Occurs at Two Specific Lysine Residues (Lys-244 and Lys-263)—To test whether or not PLAG1 is SUMOylated, co-transfection experiments were performed. Therefore, COS7 cells were co-transfected with PLAG1 cDNA together with DNA encoding Myc-tagged SUMO-1 or a DNA encoding a Myc-tagged SUMO-1 mutant form, missing the 2 C-terminal glycine residues, as a negative control. Since these 2 glycine residues are necessary for the attachment of SUMO-1 to its target proteins, it should be noted that this mutant form of SUMO-1-Myc cannot bind to its targets anymore. Such transfections were also performed in the presence or absence of the different PIAS proteins encoded by corresponding PIAS DNA constructs. Using anti-PLAG1 antibodies, we observed slower migrating bands in SDS-PAGE, which presumably correspond to SUMO-modified PLAG1 proteins. Western blot analysis revealed two band shifts in the case of PLAG1, pointing toward a double SUMO modification of this protein, whereas band shifts remained absent in cells co-expressing PLAG1 with a mutant form of SUMO-1-Myc (Fig. 2). The consensus SUMOylation sequence is defined as ψ-Lys-Xaa-Glu, in which ψ is a large hydrophobic amino acid, most frequently isoleucine or valine, and Xaa can be any amino acid (14). Screening (www.abgent.com/sumoplot.html) of the PLAG1 sequence revealed three potential SUMOylation sites with high probability, containing Lys-244, Lys-263, or Lys-353. To test the relevance of these sites, the following single mutants, namely PLAG1-K244R, K263R, and K353R, the various double mutants of these, and also the triple mutant were generated. By performing co-transfection experiments using PLAG1
DNA constructs encoding these mutant forms, we established that lysine residues Lys-244 and Lys-263 are genuine targets for SUMOylation of PLAG1 (Fig. 3). Moreover, the K244R mutation seems to have a more profound effect on PLAG1 SUMOylation than the K263R mutant since the first mutation led to the disappearance of both band shifts, pointing to a non-SUMOylated protein, whereas in the case of the K263R-mutated PLAG1, one strong band shift remained detectable (Fig. 3). Upon introduction of both of these two mutations in PLAG1, no detectable levels of SUMOylation could be observed using Western blot analysis. Finally, mutation of the lysine on position 353 did not seem to have any effect since double SUMOylation of PLAG1 remained detectable as two band shifts.

Direct reprobing of the Western blots with antibodies to SUMO to establish SUMOylation of PLAG1 revealed a big smear of the wide variety of SUMOylated proteins (data not shown), which could be anticipated on the basis of the widespread occurrence of SUMOylation in the cell. Therefore, and to independently confirm that PLAG1 physically interacts with SUMO-1-Myc in vivo, GST pull-down experiments were first performed with nuclear and cytoplasmic extracts of COS7 cells expressing GST-PLAG1 wild-type or one of its mutant forms (K244R, K263R, or K244R/K263R) together with SUMO-1-Myc as a purification step. Proteins interacting with GST-PLAG1 were subsequently size-fractionated by SDS-PAGE, and specific interaction with SUMO-1-Myc was revealed in Western blot analysis using anti-Myc antibodies. GS beads pulled down two polypeptides that were also recognized by the anti-Myc antibodies and only present in the nuclear extracts of cells in which GST-PLAG1 and SUMO-1-Myc were co-expressed (Fig. 4A). When the mutant form GST-PLAG1-K263R was expressed instead of the wild-type form, the fastest migrating of these two polypeptides could also be detected in the nuclear extracts. Under the same conditions, no anti-Myc immunoreactive polypeptides were visualized when GST-PLAG1-K244R or GST-PLAG1-K244R/K263R was co-expressed with SUMO-1-Myc. As expected, no anti-Myc immunoreactive polypeptides could be detected when wild-type GST-PLAG1 or one of its mutant forms was co-expressed with SUMO-mutant and also not when GST-PLAG1 or SUMO-1-Myc was expressed alone (Fig. 4A). As a control, the various expression levels of GST-PLAG1 fusion proteins were compared (Fig. 4B). It should be noted that the level of expression of the wild-type fusion protein was consistently higher when compared with those of the mutant proteins.

To address the question as to whether the bands detected in the nuclear extracts by anti-Myc antibodies migrate at the same position as the bands detected with anti-PLAG1 antibodies, we performed with nuclear and cytoplasmic extracts of COS7 cells transfected with GST-PLAG1 wild-type or one of its mutant forms (K244R, K263R, or K244R/K263R) together with SUMO-1-Myc inserted in DNA constructs. To evaluate SUMOylation in these experiments, DNA encoding SUMO-Myc or the mutant form of SUMO was co-transfected. As a negative control, the PLAG1 double mutant K244R/K263R was expressed instead of the wild-type form, the fastest migrating of these two polypeptides that were also recognized by the anti-Myc antibodies migrate at the same position as the bands detected with anti-Myc antibodies were present in the nuclear extracts using GS beads. Upon size fractionation of the selected GST-PLAG1 proteins by SDS-PAGE and subsequent blotting onto nitrocellulose paper, the selected proteins were first assayed with anti-Myc antibodies to detect SUMOylated (SUMO-1-Myc) PLAG1. As expected, the same results were obtained as shown in Fig. 4A (Fig. 4C, upper panel). Upon stripping of the blot and reprobing with anti-PLAG1 antibody, the same bands were visualized at the same position as with the anti-Myc antibodies; furthermore, the GST-PLAG1 band was detected (Fig. 4C, lower panel). It should be noted that the signals of the SUMOylated GST-PLAG1 proteins with the anti-PLAG1 antibody were somewhat weaker than those obtained with the anti-Myc antibody; to more clearly visualize the biSUMOylated band, a longer exposure was required (data not shown). These results clearly demonstrate that the SUMOylated PLAG1 bands detected with either anti-Myc or anti-PLAG1 migrate at the same positions.

Enhancement of SUMOylation of PLAG1 by Co-expression of Pias Proteins—Next, we investigated whether PLAG1 SUMOylation could be enhanced by co-expressing Pias proteins. Therefore, each of the different Pias family members (Pias1, Pias3, Piasy, Piasxα, and Piasxβ) was co-expressed with PLAG1 upon transfection of the appropriate DNA constructs. To evaluate SUMOylation in these experiments, DNA encoding SUMO-Myc or the mutant form of SUMO was co-transfected. As a negative control, the PLAG1 double mutant K244R/K263R was included in the assay (Fig. 5). The results reveal an increase in SUMOylation in the presence of the Pias proteins. In the case of co-expression of Pias1, PLAG1 wild-type, and SUMO-Myc, even a third band shift is visible (Fig. 5, arrowhead). Furthermore, single and double SUMO modifications of PLAG1, resulting from the presence of an endogenous pool of SUMO in the cell (thus, without co-transfection of DNA encoding SUMO-Myc), became visible on the blot as bands that each time migrated somewhat faster than the bands corresponding to the single and double SUMO-1-Myc-modified ver-
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SUMOylation Decreases the Transcriptional Activity of PLAG1—PLAG1 possesses transcriptional activity by binding to a bipartite DNA sequence containing a core (GRGGC) and cluster (GGG) separated by 6–8 random nucleotides (3). In view of our findings that PLAG1 is modified by SUMO-1, the functional significance of this covalent protein modification was therefore studied in the context of transcriptional activation. Both the single mutants (K244R and K263R) and the double mutant were compared with the wild-type PLAG1 protein with respect to their ability to activate the expression of luciferase. For this purpose, an appropriate reporter construct was used that contained three copies of the bipartite PLAG1 consensus DNA-binding site (5WT2)TKluc (8)). For this purpose, HEK293 cells were co-transfected with the (WT2)TKluc reporter plasmid DNA. Experiments were performed at least two times in duplicate, and expression levels of the various PLAG1 proteins (wild-type and mutants) were normalized based on Western blot analysis (data not shown). The results (Fig. 7A) indicate that SUMOylation seems to inhibit the transplaional forms of GST-PLAG1 resulting from SUMOylation by endogenous SUMOylation and the mono- and biSUMO(Myc)ylated forms resulting from the GST-PLAG1 SUMOylated forms by SUMO-1-Myc. The results with the various mutants were as expected from our previous co-expression studies (Figs. 4 and 5). To investigate whether the same endogenously SUMOylated PLAG1 bands detected with anti-SUMO-1 can also be detected by anti-PLAG1, the blots were treated to remove the anti-SUMO antibody and reprobed with anti-PLAG1 antibody. The results clearly show that two endogenously SUMOylated bands can be detected by anti-PLAG1 and that they migrate at the same positions as those detected with the anti-SUMO-1 antibodies (Fig. 6, lower arrow, lower panel). GST-PLAG1 is also clearly detected (Fig. 6, lower arrow, lower panel). Similarly, as already stated before, the signals of the SUMOylated PLAG1 proteins with the anti-PLAG1 antibody are somewhat weaker than those obtained with the anti-SUMO antibody; to clearly visualize the biSUMOylated band, a longer exposure was required (data not shown). Altogether, these data convincingly demonstrate that PLAG1 is subject for SUMOylation by endogenous SUMO-1.

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activation capacity of PLAG1 about 9-fold. Interestingly, the mutation of the lysine residue at position 244 into an arginine, and thus the disabling of that SUMO acceptor site, has a larger effect on the transactivation capacity of PLAG1 than mutating the lysine at position 263. The double mutant form (K244R/K263R) was also studied in the presence and absence of Myc-tagged SUMO-1 in this luciferase assay. Consistent with the observed lack of detectable SUMOylation, there was no difference in luciferase activity in these cases. It should also be noted that there was no clear difference in the observed luciferase activity when PLAG1 was assayed in the presence or absence of Myc-tagged SUMO-1. This is probably due to endogenous PLAG1 SUMOylation because of the presence of endogenous SUMO. Similarly, no difference was observed in the luciferase activity when PLAG1 was assayed in the presence or absence of non-binding SUMO-1-MUT, which can also be explained by endogenous SUMOylation. Support for this hypothesis could be found by assaying PLAG1-induced luciferase activity upon removal of SUMO using a SUMO-specific protease. For this purpose, we analyzed the effect of co-expressing SSP3,
a protease that cleaves off SUMO from its substrates (Fig. 7B) (15). In line with the proposed explanation, the relative induction of the (WT2)3TKluc reporter by PLAG1 was much higher when SSP3 was co-expressed (Fig. 7B).

SUMOylation Has No Effect on the Subnuclear Localization of PLAG1—It has been shown that SUMO modification can influence the localization of proteins to specific subnuclear structures, namely the so-called promyelocytic leukemia protein (PML)-containing nuclear bodies. However, SUMO conjugation is not always necessary for the delivery of proteins to these structures (16). Some examples are PML itself (17), homeodomain-interacting protein kinase 2 (HIPK2), translocation-Ets-leukemia (TEL), and p53-related p73α (18). On the other hand, although recruitment of transcription factors to subnuclear structures might be the critical determinant for the regulation of transcriptional activity of these proteins, SUMOylation could simply be a consequence of sequestration, as has been suggested for lymphoid-enhancing factor 1 (LEF1) (19). To investigate whether SUMOylation similarly affects nuclear localization of PLAG1, we compared the subcellular localization of wild-type PLAG1-GFP fusion proteins to the localization of the corresponding mutant forms (i.e., K244R, K263R, or K244R/K263R, as described above). Using fluorescence microscopy, the localization of PLAG1-GFP, wild-type or mutant, expressed alone, co-expressed with SUMO-Myc or the previously mentioned mutant form of SUMO-Myc, or co-expressed with the PIAS family members, was studied in COS-7 cells. Under these various experimental conditions, a diffuse and equal staining of the complete nucleus was consistently observed (data not shown). For example, triangles indicate endogenous SUMOylation of PLAG1 when PIASxα is co-expressed. In the case that PIAS1, PLAG1 WT, and SUMO-Myc are co-expressed, even a third band shift (arrowhead) is visible. Moreover, PIAS1 was clearly able to cause SUMOylation of the double mutant form of PLAG1, co-expressed with SUMO-Myc (star). A similar band shift is present in the case of PIASxα, although one should notice the higher total amount of protein loaded (star).

Fig. 5. PIAS protein family members play a role as E3 ligases in the SUMOylation of PLAG1. Western blots, obtained by transferring proteins onto nitrocellulose membranes after size fractionation on 10% polyacrylamide gels, were treated first with rabbit polyclonal anti-PLAG1 antibody PFM 195 (1:3000) directed against the N-terminal part of PLAG1 and subsequently with horseradish peroxidase-conjugated swine anti-rabbit IgG (1:1000). Immunoreactive proteins were visualized using Western Lightning chemiluminescence reagent Plus (PerkinElmer Life Sciences). DNA encoding for the different PIAS family members (PIAS1, PIAS3, PIASxα, PIASxβ, and PIASxγ, as indicated under the respective lanes in the figure) was co-transfected each with wild-type PLAG1 DNA (P1 WT) together with DNA encoding SUMO-Myc or the mutant form of SUMO (SUMO-MUT). As a control, the PIAS proteins and SUMO-Myc were co-expressed in the absence of PLAG1. As a negative control, the PLAG1 double mutant (K244R/K263R) (P1 MUT) was included in the assay. At the left part of the figure, the two band shifts (arrows), occurring when PLAG1 WT is co-expressed with SUMO-Myc, are shown. The blot reveals an increase in SUMOylation of PLAG1 due to overexpression of the various PIAS proteins, as indicated. Also, the modification of PLAG1 with endogenously expressed SUMO becomes visible under such experimental conditions. For example, triangles indicate endogenous SUMOylation of PLAG1 when PIASxα is co-expressed. In the case that PIAS1, PLAG1 WT, and SUMO-Myc are co-expressed, even a third band shift (arrowhead) is visible. Moreover, PIAS1 was clearly able to cause SUMOylation of the double mutant form of PLAG1, co-expressed with SUMO-Myc (star). A similar band shift is present in the case of PIASxα, although one should notice the higher total amount of protein loaded (star).
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**DISCUSSION**

To get more insight into the molecular context that allows a transcription factor to modulate gene expression as part of a greater protein complex, or more in general, how its transcriptional capacity is regulated, it is important to know its interaction partners. Numerous studies have also pointed toward the importance of post-translational modifications such as phosphorylation, ubiquitination, and SUMOylation (20).

This study is based on the initial observation from yeast two-hybrid assays that the transcription factor encoded by the Plagl2 proto-oncopogene interacts with several members of the PIAS protein family as well as with UBC9. From GST pull-down experiments, it appeared that all members of the PLAG protein family interacted with several members of the PIAS protein family. To study the relevance of this interaction, we focused on PLAG1, the founding member of the family. Except for PIAS3, for which the interaction was hardly detectable in GST pull-down experiments, it appeared that all members of the PIAS family interacted with similar affinity with PLAG1. It should be noted that the PIAS proteins used in these studies were derived from different species. However, these species differences do not seem to qualitatively affect the interactions with the various PLAG proteins. This is not fully unexpected, in light of their highly conserved amino acid sequences.

The observed interaction between PLAG1 and PIAS members was of interest in light of the functions of these PIAS proteins. The first member of the PIAS family, PIAS1, was originally identified in a yeast two-hybrid screening using a fragment of signal transducers and activators of transcription 1 (STAT-1) as bait (21). STATs in general are a family of transcription factors that mediate cytokine and growth factor signals culminating in various biological responses. When expressed in mammalian cells, PIAS1 and PIAS3 inhibit STAT-1 and STAT-3-mediated gene activation, respectively (21). Also, PIASy was later identified as a transcriptional co-repressor of STAT-1. This suggested that different PIAS proteins could repress STAT-mediated gene activation (22). Furthermore, since these initial findings, PIAS proteins have also been linked to other biological phenomena with no apparent connection to STAT proteins, such as induction of apoptosis, modulation of ion channels, transcriptional modulation of hormone-dependent nuclear receptors, and interaction with RNA helicase (23). Furthermore, some PIAS proteins seem to play a role as an E3 ligase in the SUMOylation of a variety of proteins, including several transcription factors (24, 25). The multistep process of SUMOylation has been reviewed in depth recently (for reviews, see Refs. 14, 26, and 27). Although target proteins can at least transiently interact with UBC9, which is known to act as an E2 enzyme in covalently modifying a variety of proteins with SUMO, in vitro studies show that E3 ligases are often needed for an efficient conjugation, as they might (i) increase the affinity of UBC9 for a specific target, (ii) stabilize the UBC9-target interaction, (iii) help to orientate the acceptor lysine, or (iv) contribute mechanistically to conjugation (27). For example, Ran-binding protein 2 (RanBP2) stimulates SUMO modification of RanGTPase-activating protein 1 (Ran-GAP1) and Sp100 but not of p53 and thus confers substrate specificity to the SUMOylation reaction (28). Recently, several reports indicated that in addition, PIAS proteins may act as E3 ligases in the SUMO pathway (24, 25). Similar to a large class of E3 ubiquitin ligases, the PIAS proteins contain a RING finger motif that is required for their activity in stimulating SUMOylation (28).

In this perspective, the possibility was raised that PLAG1 could be post-translationally modified by SUMO. Further support for this was deduced from the observation that a family member of PLAG1, i.e. mouse Plagl2, was found to interact with UBC9, the E2 enzyme of the SUMOylation pathway. The results from our studies reveal that PLAG1 does indeed undergo this covalent modification. This was firmly established by our observation that the bands detected by anti-SUMO antibodies were also detected by anti-PLAG1 antibodies, indicating that they constitute the same protein forms of PLAG1. Moreover, the PLAG1 protein seems to be SUMOylated at least at two different positions, namely Lys-244 and Lys-263. Of these, site Lys-244 seems to be the most important one. If the lysine residue at position 244 was mutated into an arginine, PLAG1 SUMOylation became undetectable on Western blots,
whereas this was not the case when only mutation K263R was introduced. In this latter case, the first band shift, caused by mono-SUMOylation of PLAG1 at site Lys-244, remained clearly visible, whereas the second band shift disappeared, apparently due to the absence of bi-SUMOylation at Lys-244 and Lys-263. Since covalent attachment of one protein to another can alter the conformation of the target protein, one explanation for the difference in the levels of SUMOylation between both mutations could be the conformational change of PLAG1. The SUMO consensus sequence containing Lys-263 could become (more) accessible for SUMOylation after PLAG1 is SUMOylated at site Lys-244.

Secondly, it should be emphasized that SUMOylation studies could be carried out solely on exogenously expressed PLAG1 proteins. PLAG1, a developmental regulated gene, is detectable during embryonic development by Northern blot analysis; however, immunological detection of the PLAG1 protein is rather difficult even in these stages. After birth, PLAG1 transcripts cannot be detected in normal tissues by Northern blot analysis, nor can the PLAG1 protein be detected by immunoprecipitation or Western blot analysis; the very low levels of PLAG1 transcripts can be detected only by multiple rounds of reverse transcriptase-PCR. In particular tumors, such as pleomorphic adenomas of the salivary glands and lipoblastomas, PLAG1 expression is up-regulated as a result of promoter swapping (often resulting from a chromosome translocation), and the levels of expression of PLAG1 vary, depending on the particular promoter and expression control elements placed upstream of the PLAG1 gene in such tumors. Although studying SUMOylation in tumor tissue was considered, this option was abandoned since it does not reflect SUMOylation of PLAG1 in normal tissue. Because of all these reasons, PLAG1 SUMOylation was studied using exogenous expression.

Co-expression of the various PIAS proteins markedly increased SUMOylation levels. In the case of co-expression of wild-type PLAG1 with PIAS1 and SUMO-1-Myc, even a third band shift became clearly visible. Under these conditions,
PIAS1 was also clearly able to mono-SUMOylate the double mutant form of PLAG1 (K244R/K263R), SUMOylation of which is normally not detectable. In the case of PIASy, a similar band shift was observed but under the conditions of a higher level of protein loaded, suggesting a lower efficiency of PIASy with respect to PIAS1. As a first explanation, this might indicate the presence of a third SUMO consensus site in our protein of interest. However, we failed to distinguish any SUMO-Myc-modified PLAG1 when the double mutant form of PLAG1 was co-expressed with SUMO-1-Myc and any other PIAS family member, apart from PIAS1 and PIASy. A second explanation could be the presence of poly-SUMOylation, although this chain formation was mostly reported in the case of SUMOylation with SUMO-2/SMT3α/Sentrin-3 or SUMO-3/SMT3β/Sentrin-2. SUMO-2 and SUMO-3 are related to SUMO-1 but are apparently functionally distinct (31). Although in mammalian cells the majority of the SUMO-1 proteins exist in their conjugated form, the SUMO-2 and SUMO-3 isoforms exist primarily as free proteins that are subjected to rapid conjugation after cellular stress (26). Their sequences possess functional SUMO consensus modification sites (ωKXE) in the N-terminal region. As a consequence, SAE1/SAE2 and UBC9 catalyze the formation of polymeric chains of SUMO-2 and SUMO-3 in vitro and SUMO-2 chains in vitro, whereas these multimeric forms were absent in the assays in which the mutant forms K11R-SUMO-2 or K11R-SUMO-3 were expressed (31). However, a recent study on the SUMOylation of RanBP2, a nucleoporin with E3-like activity and itself a target for SUMOylation, provides strong evidence that, besides SUMO-2 and SUMO-3 formation, SUMO-1 chain formation also exists (32). A third and the most probable explanation, however, is that lysine residues in PLAG1, other than Lys-244 and Lys-263, are covalently modified with SUMO-1, only under the specific condition of co-expressing SUMO-1 with particular enzymes of the SUMOylation pathway. It remains therefore to be established whether these lysines have physiological significance. The phenomenon of additional SUMOylation has already been reported for Smad4, an intracellular effector of the transforming growth factor-β signaling pathway. Briefly, the K113R/K159R double mutant form of the Smad4 protein showed almost no detectable SUMOylation in Western blot experiments. Nevertheless, co-expression of UBC9 and PIASxα did result in SUMOylation of this double mutant, pointing toward the use of alternative lysines as target residues for SUMOylation under these specific conditions. Moreover, co-expression of UBC9 and Smad1, -2, or -3, which are normally not SUMOylated, facilitated SUMOylation of these proteins, albeit to a much lesser extent than in the case of Smad4 (20).

It should also be noted that, when PLAG1 was co-expressed with PIAS, the covalent attachment of endogenous SUMO-1 that is already present in the cell could be clearly visualized in multiple experiments. Such modification of PLAG1 with endogenously expressed SUMO-1 was most pronounced in the cases in which PIAS1 or PIASy was co-expressed. Furthermore, by simply co-expressing PIAS1, PIASxα, or PIASxβ with PLAG1, there was even no need to express exogenous SUMO-1 to observe the double SUMOylated form of PLAG1. However, the generated band shifts clearly migrated faster than the bands corresponding to the Myc-tagged SUMO-modified version of PLAG1. This can be explained by the absence of the Myc tag. Altogether, these results clearly establish that the transcription factor PLAG1 is SUMOylated at least at two specific sites. There are already other examples of proteins that play a role in the transactivation of several genes and for which PIAS family members function as E3 SUMO ligase. Examples include PIAS1 as E3 ligase for p53 (33), PIASy for LEF1 (19, 24), PIAS1 and PIASxα for the androgen receptor (21, 34), and PIASy and PIASxα for Smad4 (20).

Modification of proteins with SUMO is more and more recognized as an important regulatory process in a diverse set of cellular pathways as it might influence the stability, the subnuclear localization and, in the case of transcription factors, the transcriptional capacity of target proteins (for reviews, see Refs. 26, 35, and 36). Our studies demonstrate that SUMOylation has an apparent inhibitory effect on the transcriptional capacity of PLAG1. By mutating both SUMO consensus sequences in PLAG1, the transactivation capacity of this protein is significantly increased. Moreover, Lys-244 is functionally more important for the regulation of the transactivation capacity of PLAG1 since mutation of this residue (K244R) has a more pronounced effect on this transactivating potential than the insertion of the single mutation K263R. It is even comparable with the effect caused by the double mutation K244R/K263R. This is in accordance with the fact that Lys-244 seemed to be the most important target for SUMOylation when analyzed in Western blot experiments, as described above. Two remarks need to be made here. As a first remark, it should be noted that SUMOylation is a highly dynamic and fully reversible modification. A family of cystein proteases specifically hydrolyzes SUMO isopeptide bonds. For example, mammals have at least nine SUMO proteases (SENP), localized in different subcellular compartments, such as at the PML nuclear bodies (SENP1), the cytoplasm (SENP6), the nucleolus (SENP3), or the nuclear pore (SENP2) (37). In our studies, the enzymatic activity of SUMO-specific protease 3 was used to independently confirm the effect of SUMOylation on the transactivating capacity of PLAG1, demonstrated in our mutant studies. Another option to investigate the effect of the selective abolishment of SUMOylation of PLAG1 on its transcriptional activity would be an experimental approach using the RNA interference technology. However, since PLAG1 is SUMOylated by all PIAS family members tested, although with somewhat varying efficiencies (Fig. 5), all PIAS members should be targeted in such an experimental setting. Furthermore, conditions should be optimized in such a way that all PIAS members and also other E3 enzymes are effectively taken out. The elegance of the SSP3 protease approach is that SSP3 targets SUMOylation of PLAG1 by all PIAS members and other possibly unknown E3 enzymes and, therefore, aims at the same objective as the RNA interference approach. As a second remark, one has to keep in mind that expression of mutant SUMO-1 does not lead to detectable SUMOylation of PLAG1, as already mentioned above. This is not unexpected since mutant SUMO-1 cannot bind to PLAG1 and, furthermore, levels of SUMOylated PLAG1 resulting from always present endogenous SUMO-1 are below detection in the absence of PIAS proteins to enhance SUMOylation. In the presence of PIAS proteins and mutant SUMO-1, however, SUMOylation resulting from endogenous SUMO-1 is clearly detected (for instance, Fig. 5). Thus, expression of mutant SUMO-1 does not abolish SUMOylation of PLAG1 by endogenous SUMO-1. Therefore, there is always SUMOylated PLAG1 present under these experimental conditions, and the levels are most likely similar to the levels in the absence of mutant SUMO-1. In the presence of PIAS, levels of SUMOylated PLAG1 are enhanced to the extent that they are just detectable. Thus, in the context of transcriptional activation/repression, expression of mutant SUMO-1 does not affect SUMOylation and is therefore not likely to affect the transcriptional activity of PLAG1 since endogenously SUMOylated PLAG1 is always present. Two elegant ways to affect SUMOylation of PLAG1 are to generate mutants for which SUMOylation is blocked because the SUMOylation sites have been
altered or, alternatively, use an enzyme that removes SUMO-1 from SUMOylated proteins. We have investigated both approaches, and in each case, transcriptional activity of PLAG1 appeared to be affected.

Based on the literature, the influence of modifying a transcription factor with SUMO-1 on its transcriptional activity is not necessarily a direct effect but can also be an indirect effect, caused by sequestration of the modified forms to specific sub-nuclear compartments. This has been reported, for example, in the case of LEF1, a transcription factor that interacts with PIASy and, as a consequence, accumulates in specific nuclear domains, called PML nuclear bodies (26). However, we could not demonstrate similar subnuclear accumulation of PLAG1 resulting from its SUMOylation. Altogether, our observations that the two SUMOylation-defective mutants of PLAG1, i.e. K244R and K244R/K263R, show enhanced transcriptional activation capacity in a similar way as enzymatic de-SUMOylated wild-type PLAG1 thus suggest a direct effect of SUMOylation on this activity. However, despite the fact that no detectable differences in subcellular localization of the PLAG1 mutants when compared with wild-type PLAG1 could be observed, the impact of subtle changes cannot be fully ruled out.

In conclusion, our results establish that PLAG1 is SUMOylated and that, as a direct result, its transactivating capacity is clearly repressed. Although the precise physiological impact of this post-translational modification on the functioning of the PLAG1 transcription factor remains to be established, it is tempting to speculate that SUMOylation might play a regulatory role in the dissociation of the transcriptional complexes of which PLAG1 is an integral part. A similar role was recently argued that SUMOylation of this protein changes its conformation or positioning, making the sister chromatid cohesion complex permissive for dissolution (38).

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