Modification of GATA-2 Transcriptional Activity in Endothelial Cells by the SUMO E3 Ligase PIASy

Tae-Hwa Chun, Hiroshi Itoh, Lalitha Subramanian, Jorge A. Iñiguez-Lluhí, Kazuwa Nakao

Abstract—GATA sequences are required for the optimal expression of endothelial cell–specific genes, including endothelin-1 (ET-1). We have identified PIASy in a search for new GATA-2 interacting proteins that can regulate GATA-2–mediated endothelial gene expression. Notably, among the cell populations comprising vascular walls, PIASy mRNA is selectively expressed in endothelial cells, and its expression can be regulated by angiogenic growth factors. We show that GATA-2 is covalently modified by small ubiquitin-like modifier (SUMO)-1 and -2 and that PIASy, through its E3 SUMO ligase activity, preferentially enhances the conjugation of SUMO-2 to GATA-2. Through a functional analysis, we demonstrate that PIASy potently suppresses the activity of the GATA–2-dependent human ET-1 promoter in endothelial cells. The suppressive effect of PIASy requires the GATA-binding site in the ET-1 promoter and depends on its interaction with GATA-2, which requires both N-terminal (amino acids 1-183) and C-terminal (amino acids 414-510) sequences in PIASy. We conclude that PIASy enhances the conjugation of SUMO-2 to GATA-2 and that the interaction of PIASy with GATA-2 can modulate GATA-mediated ET-1 transcription activity in endothelial cells through a RING-like domain-independent mechanism. (Circ Res. 2003;92:1201-1208.)

Key Words: GATA-2 ■ endothelin-1 ■ SUMO ligase ■ PIAS family

GATA-2 is a member of the GATA family (GATA-1 to -6) of transcription factors and plays a critical role in hematopoietic and cardiovascular development by regulating the transcription of key cell-type specific target genes. GATA-2 is expressed abundantly in primitive hematopoietic cells, and its level gradually declines during their maturation into different blood cell types. Moreover, GATA-2 is expressed in adult endothelial cells, where it plays key roles in the transcriptional regulation of endothelial-specific genes containing GATA-binding sites in their promoter regions.

Endothelin-1 (ET-1) is a potent vasoconstrictor that is expressed exclusively in endothelial cells. A GATA-binding site located in the promoter of the human ET-1 gene is necessary for the optimal expression of ET-1 in endothelial cells. Several studies have also demonstrated the significance of GATA-binding sites for the optimal expression of other endothelial-specific genes, such as endothelial nitric oxide synthase, von Willebrand factor, KDR, and platelet-endothelial cell adhesion molecule-1.

It has been postulated that coactivators or corepressors of GATA-2 could influence GATA-2–regulated expression of endothelial-specific genes and contribute to their complex regulation. In a search for new molecules that can regulate GATA-2 function in endothelial cells, we have identified PIASy as a protein that interacts with GATA-2. PIASy was originally cloned as a member of the PIAS family of regulators of STAT function. Recent reports have highlighted the ability of PIAS family members to act as small ubiquitin-like modifier (SUMO) ligases. We found that GATA-2 is modified by SUMO proteins and that PIASy enhances the extent of SUMOylation. Moreover, by examining the effect of PIASy on the promoter activity of the human ET-1 gene, we found that PIASy strongly represses ET-1 promoter activity through its interaction with GATA-2.

Materials and Methods

Plasmids

The human GATA-2 cDNA (1.7 kb) was obtained by reverse transcriptase–polymerase chain reaction (RT-PCR) from HEL cells. Yeast expression vectors are derivatives of the pBTM116 (Paul Bartel and Stanley Fields). N-terminal and C-terminal fragments of hGATA2 encoding aa 1-475 and 275-475 were subcloned into pBTM116 (pLexA-GATA2 and pLexA-GATA2ΔN). The expression vector for LexA-fused Val14 RhoA (pBTM-Val14-RhoA) was obtained from Naoki Watanabe (Kyoto University). The GATA-2 cDNA was subcloned into pcDNA3.1-V5-His (Invitrogen). The pACT2-PIASy vector corresponds to one of the full-length isolates obtained through our yeast two-hybrid screening. The FLAG-tagged PIASy expression vector, pFLAG-PIASy, was constructed by inserting a SalI/BglII fragment of pACT2-PIASy into pFLAG-CMV-2 (Sigma). Expression vectors for the deletion mutants of PIASy, pFLAG-PIASyΔN (deletion of nt 0-472), pFLAG-PIASyΔM (nt 549-1242), and pFLAG-PIASyΔC (nt 669-1533) were created by restriction enzyme digestion and religation. Three copies of the SV40 nuclear localization signal (D KKRRKKV) were inserted between the FLAG tag and PIASyΔC to create pFLAG-NLS-PIASyΔC. Inserting the SalI/BglII fragment of pACT2-PIASy into
pGEX4T3 (Pharmacia) yielded pGST-PIASy. pcDNA3-HA-SUMO-1 and pcDNA3-HA-SUMO-2/SMT3B were gifts of Kim Orth (UT Southwestern University, Dallas, Tex).16 The human ET-1 promoter (bp +204 to +180) was PCR amplified from human genomic DNA using forward (5′-CTGCCCTGCCAGTCTGAGC-3′) and reverse (5′-CGGGTTCCTCAGATCCTAAA-3′) primers (WT ET-1 promoter). Mutagenesis of the GATA and AP-1 sites was achieved by PCR and changed the GATA and AP-1-binding sequences 5′-TTATC-3′ and 5′-GTTAATA-3′ to 5′-GATACT-3′ (GATA-mut) and 5′-TTAATTA-3′ (AP-mut) respectively. The promoter fragments were ligated into pGL2 basic (Promega) to yield (pGL-wild ET, pGL-GATA-mut ET, and pGL-AP-mut ET, respectively).

**Yeast Two-Hybrid Screening**

The yeast reporter strain L40 was transformed sequentially with pLexA-hGATA2ΔN and a human placenta matchmaker cDNA library (Clontech) that produces fusion proteins with the GAL4 activating domain. Candidate clones were identified as histidine prototrophs and as positive for Lac-Z. To confirm the specificity of the association, candidate clones were cured of bait plasmid and assayed for histidine prototrophy and as positive for Lac-Z. To confirm the specificity of the association, candidate clones were cured of bait plasmid and assayed for histidine prototrophy and as positive for Lac-Z. To confirm the specificity of the association, candidate clones were cured of bait plasmid and assayed for histidine prototrophy and as positive for Lac-Z. To confirm the specificity of the association, candidate clones were cured of bait plasmid and assayed for histidine prototrophy and as positive for Lac-Z. To confirm the specificity of the association, candidate clones were cured of bait plasmid and assayed for histidine prototrophy and as positive for Lac-Z. To confirm the specificity of the association, candidate clones were cured of bait plasmid and assayed for histidine prototrophy and as positive for Lac-Z.

**Immunoprecipitation, Western Blot, and Immunofluorescence**

Transfection of COS 1 cells with empty pFLAG-CMV2 vector, pFLAG-PIASy, -PiasyΔM, -PiasyΔC, -PiasyΔN, or NLS-PiasyΔC together with pcDNA3.1-V5-His vector or pcDNA-GATA2-V5-His was performed with Lipofectamine (Gibco). Cell lysates were prepared 48 hours after transfection in lysis buffer (20 mmol/L Tris-Cl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100) with protease inhibitor cocktail (Sigma). Expression of GATA2-V5 and FLAG-tagged proteins was analyzed by Western blotting using anti-V5-HRP antibody (Invitrogen) or anti-FLAG Bio-M2 antibody (Sigma) followed by anti-biotin-HRP antibody, respectively. Immunoprecipitations were carried out for 1 to 2 hours at 4°C by adding 3 μg of anti-FLAG M2 antibody to cell lysates (500 μg) followed by an overnight incubation at 4°C with protein G-Sepharose (Amersham). Samples were washed four times with lysis buffer and subjected to SDS/PAGE on 7.5% gels, and V5-tagged proteins were visualized as described above.

Transfected COS 1 cells were seeded onto glass slides 24 hours after transfection. After an additional 24 hours, slides were fixed in 4% paraformaldehyde in TBS for 10 minutes and permeabilized with 0.5% Triton X-100 for 5 minutes. The slides were incubated overnight with FLAG M2 monoclonal antibody, and secondary detection was performed with Alexa Fluor 594 anti-mouse IgG (Molecular Probe). Nuclear structure and F-actin were visualized with Hoechst 33342 and Alexa Fluor 488-phalloidin (Molecular Probe). Detection was performed with Alexa Fluor 594 anti-mouse IgG (Invitrogen) or anti-HA (Covance) antibodies followed by goat anti-mouse HRP conjugate.

**SUMOylation Assay**

HEK 293T cells were transfected with pcDNA-GATA2-V5-His and either pcDNA-HA-SUMO-1 or pcDNA-HA-SUMO-2 in the presence or absence of pFLAG-PIASy using Lipofectamine Plus. Cells were harvested 36 hours after transfection in 0.7 mL of urea lysis buffer (8 mol/L ET-1 promoter (bp +204 to +180) was PCR amplified from human genomic DNA using forward (5′-CTGCCCTGCCAGTCTGAGC-3′) and reverse (5′-CGGGTTCCTCAGATCCTAAA-3′) primers (WT ET-1 promoter). Mutagenesis of the GATA and AP-1 sites was achieved by PCR and changed the GATA and AP-1-binding sequences 5′-TTATC-3′ and 5′-GTTAATA-3′ to 5′-GATACT-3′ (GATA-mut) and 5′-TTAATTA-3′ (AP-mut) respectively. The promoter fragments were ligated into pGL2 basic (Promega) to yield (pGL-wild ET, pGL-GATA-mut ET, and pGL-AP-mut ET, respectively).

**Tissue Culture and Northern Blot Analysis**

A C57BL6 mouse was killed according to Kyoto University’s Animal Rights Guidelines, and tissue RNA was isolated using Trizol reagents (GibcoBRL). Human tissue blots were obtained from Clontech. Mouse vascular endothelial cells isolated from skin dermis17 were obtained from Hedwig Murphy (University of Michigan, Ann Arbor, Mich) and characterized by von Willebrand factor, platelet-endothelial cell adhesion molecule-1, and VE-cadherin staining. Isolated mouse vascular smooth muscle cells18 homogeneously expressing smooth muscle 22α, calponin, and smooth muscle-actin were obtained from S. Filippov (University of Michigan). Skin fibroblasts were obtained from F. Sabeh (University of Michigan). Bovine carotid endothelial cells (BAECs) were isolated as previously described.19 Cells were maintained in DMEM with 10% FCS. BAECs were serum-starved for 24 hours before stimulation with 50 ng/mL human vascular endothelial growth factor (VEGF), 10 ng/mL basic fibroblast growth factor (bFGF), or 10% serum. Total RNA was prepared as above, and Northern blots were probed with a radiolabeled BglII fragment of pACT2-PIASy.

**ET-1 Promoter Luciferase Reporter Assay in BAECs and Hela Cells**

BAECs or Hela cells cultured in 12-well dishes were transfected with reporter plasmids, pGL-wild ET-1, -GATA-mut ET-1, or -AP-mut ET-1 together with the expression vectors, pFLAG-PIASy, or pcDNA-GATA2-V5. All experiments were controlled with the appropriate empty vectors. Luciferase activities were measured 48 hours after transfection according to the manufacturer’s protocol (Promega). Parallel transfections using pRL-CMV vector (Promega) and assaying for Renilla luciferase indicated that transfection efficiency did not vary by >10%.

**Electrophoretic Gel Mobility Shift Assay**

Recombinant GST-PIASy was purified from XL-1 blue cells (Stratagene) transformed with pGST-PIASy. After 8 hours of induction with IPTG, the GST-fusion proteins were purified by glutathione-Sepharose chromatography according to the manufacturer’s protocol (Amersham).

Crude mini-nuclear extracts from BAECs were prepared as described previously.20 GATA consensus (CAGCTGTGAAAAGTACTAATC) or mutant oligonucleotides (CAGCTG-TAACAGAAAAAGTCTTTAACTCT) (50 ng, Santa Cruz) were end-labeled with γ[32P]-ATP and T4 kinase (Takara Shuzo). Nuclear extracts (6 μg) were mixed with 2 μL (2 ng) of end-labeled oligonucleotide probes, 1 μg of poly(dI-dC) (Amersham), and the indicated amounts of GST-PIASy in binding buffer (10 mmol/L HEPES, 50 mmol/L KCl, 5 mmol/L MgCl2, 1 mmol/L EDTA, 5% glycerol). After a 20-minute incubation on ice, samples were resolved by electrophoresis in 0.5×TBE 7.5% polyacrylamide gels. Complexes were visualized by autoradiography.

**Results**

**PIASy Interacts With GATA-2**

To search for candidate proteins that regulate GATA-2–mediated endothelial gene expression, we used the yeast two-hybrid method to identify GATA-2 interacting proteins. Screening of 3×10⁶ colonies transformed with human placenta GAL4 activation domain fusion cDNA libraries yielded 45 candidates. Subsequent mating assays revealed that five clones exhibited a specific interaction with LexA-fused to full length GATA-2 (aa 1-475) or the C-terminal half of GATA-2 (GATA2ΔN, aa 275-475). These clones did not interact with the control LexA-Val14-RhoA fusion (Figure 1A). The entire nucleotide sequences of two clones were identical, and a BLASTN search of the GenBank database identified the insert as the full-length cDNA of human PIASy.11

**SUMOylation Assay**

HEK 293T cells were transfected with pcDNA-GATA2-V5-His and either pcDNA-HA-SUMO-1 or pcDNA-HA-SUMO-2 in the presence or absence of pFLAG-PIASy using Lipofectamine Plus. Cells were harvested 36 hours after transfection in 0.7 mL of urea lysis buffer (8 mol/L ET-1 promoter (bp +204 to +180) was PCR amplified from human genomic DNA using forward (5′-CTGCCCTGCCAGTCTGAGC-3′) and reverse (5′-CGGGTTCCTCAGATCCTAAA-3′) primers (WT ET-1 promoter). Mutagenesis of the GATA and AP-1 sites was achieved by PCR and changed the GATA and AP-1-binding sequences 5′-TTATC-3′ and 5′-GTTAATA-3′ to 5′-GATACT-3′ (GATA-mut) and 5′-TTAATTA-3′ (AP-mut) respectively. The promoter fragments were ligated into pGL2 basic (Promega) to yield (pGL-wild ET, pGL-GATA-mut ET, and pGL-AP-mut ET, respectively).
PIASy is a member of the Protein Inhibitor of Activated Stat (PIAS) family of proteins, the founding members of which were initially described as negative regulators of STAT function. PIASy contains a scaffold-associated region (SAR)-specific bipartite DNA-binding domain at amino acids (aa) 12 to 46, a RING finger-like domain at 323-368, and a serine-rich acidic domain (Ser/Ac) at 470-492, and a nuclear localization domain (NLS) at 504-510.

To confirm the interaction of PIASy with GATA-2 in mammalian cells, expression vectors for N-terminally FLAG-tagged PIASy (FLAG-PIASy) or three deletion mutants, ie, PIASy ΔM, ΔC, and ΔN (Figure 1B), were transfected into COS 1 cells together with the expression vector for GATA-2. GATA-2 was specifically coimmunoprecipitated with an anti-FLAG antibody in complex with the full-length FLAG-PIASy (Figure 1C, lane 2). The deletion of aa 183-413 encompassing the RING domain (FLAG-PIASyΔM) did not affect the interaction with GATA-2 (lane 4). In contrast, deletion of the entire C-terminal half of PIASy (Δ224-510, FLAG-PIASyΔC) or the first 157 aa (PIASyΔN) significantly reduced the interaction (lanes 5 and 6). This analysis suggests that the interaction between GATA-2 and PIASy involves the C-terminal region of GATA-2 and both amino (1-157) and C terminal (414-510) determinants in PIASy. Examination of the subcellular localization of PIASy by confocal microscopy showed that PIASy localizes to the nucleus and is enriched at the nuclear envelope. This was confirmed through costaining with the DNA dye Hoechst 33342 and visualization of the cell contour by staining for cortical actin (Figures 2A through 2E). Deletion of the N-terminal domain, which contributes to the interaction with GATA-2, did not affect the PIASy nuclear localization (Figure 2F). The PIASyΔM construct lacking the RING domain still localized to the nucleus, but the association with the nuclear envelope appeared weaker (Figure 2G). Deletion of the C-terminal half of PIASy (PIASyΔC) shifted the localization of the protein to the cytoplasm, suggesting that this region harbors a nuclear localization signal (Figure 2H). To examine whether the reduced interaction of PIASy ΔC with GATA-2 (Figure 1C) is attributable to its cytoplasmic localization, we inserted three SV40-derived nuclear localization signals between the FLAG-tag and the PIASyΔC signal (Figure 2I). This operation restored nuclear localization to PIASyΔC (Figure 2I). The interaction of this construct with GATA2, however, remained marginal (Figure 2J).

**Expression of PIASy in Endothelial Cells**

PIASy mRNA was observed ubiquitously in several mouse tissues (Figure 3A) with testis showing substantially higher levels. A similar expression pattern was observed in human tissue blots (data not shown). Given our interest in the role of PIASy in the regulation of endothelial gene expression, we examined PIASy mRNA levels in individual cell types of the vascular wall. PIASy mRNA was readily detectable in isolated vascular endothelial cells, whereas expression was much weaker in smooth muscle cells and fibroblasts (Figure 3B). This pattern of expression is similar to that of GATA2 and indicates that in the vascular wall, PIASy may play a role in regulating endothelial-specific processes. To examine the possible regulation of PIASy mRNA expression, BAECs were stimulated with VEGF, bFGF, or FCS. All three stimuli augmented expression of PIASy mRNA significantly (Figure 3C). The existence of a regulatory mechanism to upregulate PIASy suggests that this protein may alter the function of activated endothelial cells.
PIASy Enhances SUMO Conjugation to GATA-2

Recent findings suggest that PIAS family members function as SUMO ligases.13 Given that PIASy interacts with GATA-2, we examined if GATA-2 is SUMOylated and whether PIASy can promote the extent of its modification. HEK 293T cells were cotransfected with GATA-2-V5-His and HA-SUMO-1 or HA-SUMO-2 in the absence or presence of PIASy. GATA-2 was then purified under denaturing conditions via Ni-NTA chromatography. Western blot analysis demonstrated equal recovery of GATA-2 in all conditions (Figure 4A, left). In addition, minor slowly migrating bands could be detected, particularly from cells that were cotransfected with SUMO-2 and PIASy (Figure 4A, left). Probing the same samples for HA immunoreactivity demonstrated that the upper bands correspond to SUMO-conjugated GATA-2 (Figure 4A, right). Notably, the SUMO-2 conjugated form of GATA-2 was strongly enhanced by PIASy (Figure 4A, right). Probing of the crude extracts with the anti-HA antibody revealed that PIASy enhances the SUMO modification of multiple cellular proteins. As in the case of GATA-2, a stronger effect is observed for SUMO-2 (Figure 4B, right). Flag immunoblots clearly demonstrate the appropriate expression of PIASy (Figure 3B, left). These results show that GATA-2 is a target for SUMO modification and that PIASy enhances the SUMO conjugation of GATA-2, especially by SUMO-2.

Suppression of ET-1 Promoter Activity by PIASy in Endothelial Cells

We next examined whether PIASy affects GATA-2–mediated gene regulation in endothelial cells. We chose the ET-1 promoter because its activity has been well characterized and is a clear example of GATA-mediated endothelial gene regulation.7,8,23 To assess the effect of PIASy on the ET-1 promoter, we constructed luciferase reporter constructs containing the proximal promoter region (bp −202 to +180) of the human ET-1 gene, which contains single GATA (TTATCT, bp −136 to −131) and AP-1 (TGACTAA, bp −108 to −102) binding sites. Transfection of GATA-2 upregulated the ET-1 promoter activity in BAECs dose-
dependently (Figure 5A). When PIASy was cotransfected, the effect of GATA-2 on ET-1 promoter activity was no longer observed (Figure 5A). Cotransfection of increasing amounts of PIASy showed a dose-dependent suppression of ET-1 promoter activity in endothelial cells (Figure 5B). To examine the role of specific PIASy regions on the GATA-dependent transcription regulation of the ET-1 promoter, we tested the individual PIASy deletion mutants in endothelial cells. The RING-like domain deleted PIASy (PIASyΔH9004), which still interacted with GATA-2, showed the suppressive effect on ET-1 promoter activity (Figure 5C). Deletion of the entire C-terminal domain (PIASyΔH9004C) abolished the inhibitory effect on ET-1 promoter activity. As in the case of GATA-2 interaction, this lack of suppression was not attributable to the impaired nuclear localization, because shifting its localization to the nucleus by addition of three copies of the SV40 NLS (Figure 2I) did not restore inhibition of the ET-1 promoter (Figure 5C). The N-terminally deleted mutant (PIASyΔN), which appeared to interact only feebly with GATA-2, did not retain any suppressive effect on ET-1 promoter activity but rather enhanced the basal ET-1 promoter activity. The correlation between GATA-2 binding and inhibition of the ET-1 promoter suggests that the physical interaction between PIASy and GATA-2 involving both N-terminal and C-terminal regions is important in the control of ET-1 promoter activity. Furthermore, the RING-like domain is dispensable for the interaction and ET-1 promoter inhibition.

To examine the cell specificity of the PIASy-mediated suppression of the ET-1 promoter, we examined the effect of PIASy in Hela cells expressing GATA-2. The basal activity of the ET-1 promoter in Hela cells is less than one tenth the activity in endothelial cells. Contrary to endothelial cells, expression of PIASy in Hela cells did not show any inhibitory effect on the ET-1 promoter activity (Figure 5D). Similar results were obtained in COS 1 cells (data not shown). This suggests that PIASy-mediated suppression of ET-1 promoter activity is specific to endothelial cells.

**GATA Binding Site–Dependent Suppression of ET-1 Promoter Activity by PIASy**

To investigate the sites in the ET-1 promoter responsible for the repressive effect of PIASy, we mutated the GATA site (bp 136 to 131) or the AP-1 site (bp 136 to 131) in the ET-1 promoter. The basal luciferase activity of the GATA site–mutated and AP-1 site–mutated ET-1 promoters in BAECs was approximately one half and one fourth of that observed with the WT ET-1 promoter, respectively. Consistent with the results shown in Figure 4, PIASy inhibited the WT ET-1 promoter substantially (Figure 6, left). In contrast, the GATA site–mutated ET-1 promoter displayed lower activity and was resistant to the repressive effect of PIASy (Figure 6, center). Although mutation of the AP-1 binding site in the ET-1 promoter reduced overall activity, the ability of PIASy to repress was still observable in this context (Figure 6, right).

**PIASy Does Not Alter the GATA Sequence Binding Activity of Endothelial Cells**

PIAS1 and PIAS3 have been reported to inhibit the DNA-binding activity of STAT1 and STAT3.24 We therefore examined whether a similar mechanism contributes to the suppressive effects of PIASy on GATA-2 function. Nuclear extracts from BAECs were incubated with increasing amounts of GST or GST-PIASy. The specificity of the binding was confirmed by competition with a 40-fold excess of unlabeled GATA probe (lane 3). In our experiments, addition of increasing amounts of GST or GST-
In this study, we have identified an interaction between GATA-2 and PIASy and delineated the general requirements for this interaction. We have shown that PIASy expression is regulated in endothelial cells and have confirmed its SUMO E3 ligase activity. We also demonstrated that GATA-2 is covalently modified by SUMO and that PIASy enhances this modification. Notably, PIASy potently suppressed GATA-2–dependent ET-1 promoter activity in endothelial cells without altering GATA-2 DNA binding. The inhibitory effect required the same regions in PIASy implicated in its interaction with GATA-2.

Based on our two-hybrid data, both a full-length and an N-terminally–deleted GATA-2 interact with PIASy. This suggests that GATA-2 regions crucial to the interaction lie in the C-terminal region of GATA-2 (275-475 aa), which contains two zinc-finger domains. Our PIASy deletion-mutant analysis revealed that the interaction of PIASy with GATA-2 does not require its RING domain. Rather, the interaction requires both N-terminal (1-188 aa) and C-terminal (414-510 aa) sequences. The N-terminal domain of PIASy contains a scaffold-associated region (SAR)–specific bipartite DNA-binding domain, which is involved in binding to scaffold-associated regions of chromosomes.21 SARs or otherwise called matrix attachment regions are anchorage sites within chromosome loops that possess high affinity to nuclear matrix proteins,25 including lamin.26 Lamin is a major component of the nuclear lamina structure, and the DNA-binding activities of SP-1, ATF, CCAAT, C/EBP, OCT-1, and AP-1 have been reported to be enriched in these regions.27 Consistent with its SAR binding properties, we detected an interaction between PIASy and lamin C by the yeast two-hybrid assay (data not shown). Notably, a colocalization of GATA-1 and lamin C in erythroleukemia cells has been already reported.28 It is intriguing to consider that by interacting with both the nuclear lamina and GATA-2, PIASy could lead to the localization of GATA-2 in the proximity of other nuclear lamina-related transcription factors. We are presently examining this issue.

Our findings that among vascular wall cell types, PIASy is selectively expressed in endothelial cells, coupled with the observations that PIASy mRNA can be upregulated by angiogenic growth factors, indicate that PIASy and GATA-2 coexist in the endothelium and thus are likely to interact in their normal and pathophysiological context.

The PIAS family has been recently highlighted as E3 ligases for covalent conjugation of SUMO to target proteins.29 Several transcription factors have been identified as

![Figure 5. Suppression of the ET-1 promoter activity by PIASy in endothelial cells. A, BAECs were transfected with the WT ET-1 promoter luciferase reporter, and increasing amounts of pcDNA-GATA2-V5 in the absence (open columns) or presence (filled columns) of 30 ng pFLAG-PIASy. Luciferase activity was measured 48 hours after transfection. B, BAECs were transfected as in panel A, with the indicated amounts of pFLAG-PIASy and 100 ng pcDNA-GATA2-V5. C, WT ET-1 promoter activities were measured for the BAECs transfected with 30 ng of either empty vector (open bar) or expression vectors for the indicated PIASy forms together with 100 ng of pcDNA-GATA2-V5. The relative ET-1 promoter activities are normalized to the vector alone value, which was set to 100%. D, HeLa cells were transfected with the same plasmids as in B. Error bars represent 1 SD from the mean.](http://circres.ahajournals.org/)

100 ng pcDNA-GATA2-V5.
the target of PIAS-enhanced SUMOylation, including p53, c-jun, lymphoid enhancer factor 1, androgen receptor, and C/EBPα. In this study, we could demonstrate that GATA-2 is SUMOylated and that PIASy can enhance the extent of this modification, especially in the case of the SUMO-2 isoform. Human GATA-2 contains two putative SUMOylation sites, MKME (aa 221-224) and MKKE (aa 388-391), that conform to the SUMOylation consensus sequence (ΨKXE, where Ψ represents a large hydrophobic amino acid). These SUMOylation consensus sequences are conserved among other GATA family members from GATA-1 to GATA-6. Thus, SUMOylation of other GATA family members by PIAS proteins may occur in hematopoietic cells.

We have demonstrated that PIASy suppresses GATA-2-dependent ET-1 promoter activity in endothelial cells. The lack of inhibition observed in Hela and COS 1 cells indicates that the effect of PIASy on ET-1 promoter activity is selective to endothelial cells. This implies that additional endothelial-specific components beyond GATA family members from GATA-1 to GATA-6. Thus, SUMOylation of other GATA family members by PIAS proteins may occur in hematopoietic cells.

We have demonstrated that PIASy suppresses GATA-2–dependent ET-1 promoter activity in endothelial cells. The lack of inhibition observed in Hela and COS 1 cells indicates that the effect of PIASy on ET-1 promoter activity is selective to endothelial cells. This implies that additional endothelial-specific components beyond GATA-2 may be required for the inhibition of GATA-2 function by PIASy. Conversely, nonendothelial cells might contain factors that prevent PIASy from inhibiting GATA-2. Such factors could be additional proteins bridging or stabilizing the interaction between GATA-2 and PIASy or additional components of the SUMO conjugation pathway including SUMO proteases.

Our deletion analysis results indicate that both N-terminal (aa 1-183) and C-terminal (aa 414-510) portions of PIASy are required for inhibition. These regions also correspond with those required for its interaction with GATA-2. Furthermore, the inhibitory effect of PIASy on the ET-1 promoter is mediated through the GATA binding sequence. Taken together, our results imply that the suppressive effect of PIASy on GATA-2 activity is attributable to the interaction between PIASy and GATA-2.

SUMO modification of transcription factors is often associated with reduced activity, and in some cases this seems to be attributable to reduced transcriptional synergy at promoters harboring multiple binding sites for the factor. Here we showed that GATA-2 is SUMOylated, and PIASy enhances this modification. Notably, the ET-1 promoter used here harbors only a single GATA site, and in this context, the RING finger–like domain in PIASy, which is essential for its SUMO ligase activity, was dispensable for the repressive effect on the ET-1 promoter. Given that the inhibitory effect of SUMO modification of transcription factors may be restricted to promoters with multiple binding sites, it is still possible that additional RING domain–dependent effects of PIASy on GATA-2 activity could be revealed at promoters harboring multiple GATA sites. Clearly, additional research is required to address the role of SUMO modification in the function of GATA-2 and its modification by PIASy.

In conclusion, we have identified PIASy as a GATA-2 interacting protein that enhances the SUMO conjugation of GATA-2. PIASy is expressed in endothelial cells and suppresses GATA-2–mediated ET-1 promoter activity in a cell-type–specific manner. Our findings reveal an additional level of complexity in the mechanisms by which GATA-2 regulates endothelial gene expression and suggest that manipulating the activity of transcriptional modifiers such as PIASy could be exploited to control endothelial function.

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