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PIASy-Deficient Mice Display Modest Defects in IFN and Wnt Signaling

Wera Roth,* Claudio Sustmann,* Matthias Kieslinger,* Andrea Gilmozzi,* Denis Irmer,* Elisabeth Kremmer,† Chris Turck,‡ and Rudolf Grosschedl2*

Protein inhibitors of activated STATs (PIAS) represent a small family of nuclear proteins that modulate the activity of many transcription factors and act as E3 ligases for covalent modification of proteins with the small ubiquitin-like modifier (SUMO). In particular, PIASy has been shown to inhibit the activation of gene expression by the IFN-responsive transcription factor STAT1 and the Wnt-responsive transcription factor LEF1. To assess the function of PIASy in vivo, we generated and analyzed mice carrying a targeted mutation of the Piasy gene. We find that homozygous mutant mice have no obvious morphological defects and have a normal distribution of lymphocyte populations. Molecular analysis of signaling in response to IFN-γ and Wnt agonists revealed a modest reduction in the activation of endogenous and transfected target genes. Two-dimensional analysis of total proteins and SUMO-modified proteins in transformed pre-B cells showed no significant differences between wild-type mice and homozygous mutant mice. Taken together, our data indicate that PIASy has a modest effect on cytokine and Wnt signaling, suggesting a redundancy with other members of the family of PIAS proteins.

bodies (3, 20, 21, 34, 41). This observation could account for the repression of transcription factor activity without changes in DNA binding. In addition, PIASxβ has been found to interact with histone deacetylase-3, which is involved in repression of TFII-I family proteins (25). The activation function of PIAS proteins could also be accounted for by the sumoylation-dependent augmentation of protein-protein interactions. In nuclear hormone receptors, the SUMO modification sites coincide with the protein motifs that mediate synergy in the activation of reporters containing multimerized binding sites for the hormone receptors (42–44).

Genetic experiments in Drosophila indicated that the PIAS orthologue dPIAS, also known as Zimp, interacts functionally with the STAT orthologue stat92E to regulate blood cell and eye development (45). An additional role of PIAS in the regulation of chromosome structure and function was inferred from the identification and characterization of dPIAS as a suppressor of position-effect-variegation, Su(var)2-10 (46). Likewise, a yeast orthologue of PIAS proteins, termed Siz1 was identified through its genetic interaction with the condensing complex (47) and shown to act as a SUMO E3 ligase for septin proteins (14, 16). However, the function of PIAS proteins in the mouse has not yet been elucidated. We report the generation and analysis of a mouse carrying a targeted mutation of the Piasy gene. We find no gross phenotypic abnormalities, although the Mendelian ratio of viable homozygous mutant offspring is significantly reduced relative to that of homozygous multimerized binding sites for the hormone receptors (42–44).

Materials and Methods

Generation of Piasy mutant mice

A mouse genomic library from 129/Sv mice (Adashll) was screened with a Piasy CDNA probe encompassing the full-length coding sequence of mouse Piasy. A phage clone was isolated that included exons 2 through 9 of the mouse Piasy gene. A 10-kb genomic Piasy gene fragment (48), including exons 2 through 9, was excised by EcoRI and subcloned into a Bluescript vector (Stratagene, La Jolla CA). For the generation of a targeting construct a 2.1-kb genomic RI/H1 fragment, encompassing parts of intron 1, exon 1, and 217 bp of exon 2 was used in combination with a 4.2-kb genomic, PCR-amplified fragment encompassing the 3′ end of intron 2, exons 3 through 9 and the 5′ end of intron 9. A LacZ-PGKneo gene cassette was inserted in frame into a BamHI site located in exon 2 giving rise to the final targeting construct, where the 3′ 188 bp of exon 2 and 5150 bp of intron 2 were deleted (see Fig. 1). To allow positive-negative selection, a HSV-β expression cassette was inserted into the EcoI site of the Bluescript vector. For electroporation of D3 embryonic stem cells the targeting vector was linearized with SalI. Electroporation and selection of embryonic stem (ES) cells and the processing of G418-resistant ES cell clones were performed as described (49). Homologous recombination was identified by DNA blot analysis with internal, 5′ and external probes (see Fig. 1 and data not shown).

Mice

The animals used in all studies were maintained and bred according to institutional guidelines in the mouse facility of the Institute of Biochemistry (University of Munich, Munich, Germany). A PCR-based screening method was used to distinguish wild-type, heterozygous, and homozygous mutant mice using oligonucleotide primers as follows: 60–12c, 5′-GAT GCTGCTGGTGTGGTAG-3′; 60–12n, 5′-CGTACCAGTGGTTGA GAAG-3′; lacI–2, 5′-GTCGGATCTTCTCGTGGA-3′.

DNA blot analysis

Genomic DNA was isolated from ES cells and tail biopsies. Then 15 μg of each sample was digested by restriction enzymes (see Fig. 1). The fragments were separated on a 0.8% agarose gel and transferred to nylon membranes. The internal, the 5′ and 3′ external probes were labeled with [α-32P]dCTP by Random priming (Roche Diagnostics, Indianapolis, IN) and hybridized overnight at 42°C. Blots were washed to final stringency of 0.2% SDS, 0.1% SDS at 65°C, and exposed to film.

Immunoblot analysis

Total protein extracts were separated by 10% SDS-PAGE and transferred to nitrocellulose. Immunoblots were conducted by the ECL method according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Piscataway, NJ), with the Abs rat anti-PIASy (mouse) mAb.

Flow cytometry

Thymus, spleen, and bone marrow were isolated and dispersed in RPMI 1640, containing antibiotics and gluta mine with 10% heat-inactivated FCS. Cells (1 × 106) were resuspended in 100 μl of FACS buffer (PBS, 1% FCS) and incubated with Ab for 20 min. Cells were washed three times with FACS buffer, and the process was then repeated with secondary Abs as necessary. After staining, cells were resuspended in 500 μl of FACS buffer containing propidium iodine. Cells were then analyzed using a FACS-Calibur (BD Biosciences, San Jose, CA). Data generated was interpreted using CellQuest Software (BD Biosciences). Data shown in the figures are gated for lymphocytes by size and complexity and for living cells by exclusion of DNA uptake. Three-color flow cytometry was used to analyze cells that uptake propidium iodine. Directly conjugated Abs specific for mouse B220, CD43, GR1, and IgM were obtained from BD Biosciences.

β-galactosidase (β-Gal) detection of Piasy-β-Gal expressing lymphocytes

Thymus, spleen, and bone marrow cell suspensions were prepared as described above. After cell surface staining for flow cytometry, cells were resuspended in growth medium. Cells were mixed with an equal volume of 2 μM fluorescein-digalactoside (Molecular Probes, Eugene, OR) and incubated for 1 min at 37°C. After washing, the cells suspensions were diluted 10-fold with ice-cold growth medium and incubated on ice for 30–60 min. Cells were centrifuged and resuspended in 500 μl of FACS buffer and run on a FACS Calibur (BD Biosciences).

X-Gal staining of whole mount embryos

Dissected E16.5 embryos were fixed with 4% paraformaldehyde in PBS for 10 min on ice and stained for β-Gal activity by incubation in PBS containing 2 mM MgCl2, 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, and 0.4 mg/ml β-naphtho-4-chloro-3-indolyl β-D-galactoside (X-Gal) at 37°C for 2–24 h. After staining, the embryos were washed in PBS, further fixed in 4% paraformaldehyde in PBS at 4°C and cleared using a benzyl alcohol to benzyl benzoate ratio (1:2) according to standard procedures.

X-Gal staining of cryosections of mouse embryos

E14.5 and E16.5 embryos were dissected in cold PBS and dry-frozen on dry ice. Prior cryostat sectioning, embryos were embedded and oriented in tissue freezing medium (Jung; Leica, Deerfield IL). Twenty-micrometer cryosections were fixed for 15 min in 0.2% glutaraldehyde in fixing solution (PBS, pH 7.4, containing 5 mM EGTA, pH 7.3, 2 mM MgCl2) at room temperature. After washing three times for 10 min in washing solution (PBS, pH 7.4, containing 2 mM MgCl2, 0.02% Nonidet P-40), sections were stained overnight in X-Gal staining solution (washing solution, containing 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 1 mg/ml X-Gal) at 37°C. Sections were counterstained for 75 s as indicated using Nuclear Fast Red (Vector Laboratories, Burlingame, CA) and mounted in DPX (Fluka, Buchs, Switzerland).

Plasmid constructs

Plasmids encoding for full-length LEF1 have been previously described (50, 51). The pLEF-fos-luciferase vector and the pCMV-β-catenin expression vector have been previously described (52). The pRL-TK Renilla control vector (Promega, Madison, WI) was used for normalization in luciferase reporter assays.

Cell culture, transient transfections, and reporter assays

MEFs were harvested from E12.5 embryos according to standard procedures (53). Genotyping was performed as described. MEFs were cultured in high glucose DMEM supplemented with penicillin-streptomycin-glutamine and 10% FCS (Invitrogen Life Technologies, Grand Island, NY). MEFs were transfected using the Amaxa Nucleofector (Amaxa Biosystems, Cologne, Germany) and the MEF2 Nucleofector kit (programme
A23). The total DNA concentration was kept constant by adding empty vector plasmid DNA. Cells were harvested 26–28 h posttransfection in 200 μl of reporter lysis buffer, and luciferase assays were conducted according to the manufacturer’s instructions (Promega). Firefly luciferase activities were normalized against simultaneously measured Renilla luciferase activity (Promega Dual Luciferase Reporter Assay System).

**Establishment of Abelson murine leukemia virus (MuLV) transformed pre-B cell lines**

Fetal livers were isolated from E16.5 embryos and dispersed in RPMI 1640 containing antibiotics and glutamine with 10% heat-inactivated FCS by standard techniques. The Abelson MuLV was used to immortalize pools of pre-B cell populations (54).

**Splenocyte cultures**

Spleens were isolated from 3-mo-old mice and dispersed in RPMI 1640 containing antibiotics and glutamine with 10% heat-inactivated FCS by standard techniques. Cells were cultured overnight, counted, and 1.8 × 10^7 cells each were replated at a density of 2.25 × 10^6/mL. After 24 h, splenocytes were stimulated for 2 h with 500 U/ml murine IFN-γ (R&D Systems). Cells were harvested by centrifugation followed by total RNA preparation using TRIzol (Invitrogen Life Technologies).

**Bone marrow macrophage cultures**

Bone marrow was harvested from femurs of 3-mo-old animals by rinsing marrow cavities with PBS. Cells were cultured overnight in RPMI 1640 containing antibiotics and glutamine with 10% heat-inactivated FCS, and 10 ng/ml murine M-CSF (R&D Systems). Cells were counted and 2.5 × 10^7 cells per plate were seeded on new tissue culture dishes, and macrophages were expanded for 6 days using 10 ng/ml M-CSF. Expanded macrophages were stimulated for 2 h with 500 U/ml murine IFN-α (R&D Systems) and 30 ng/ml murine IFN-γ (R&D Systems). Splenocytes were harvested by centrifugation followed by total RNA preparation using TRIzol (Invitrogen Life Technologies).

**SYBR-green based real-time PCR**

The mRNA expression level of Wnt and STAT signaling target genes was measured using SYBR Green based real-time PCR. Total RNA was isolated with TRIzol (Invitrogen Life Technologies) and 2 μg of RNA were transcribed to cDNA using 200 U SuperscriptII (Invitrogen Life Technologies) and 0.1 nM oligo(dT)12–18 in a total volume of 20 μl. For one real-time reaction, 2× SYBR Green Mix (Applied Biosystems) was supplemented with 1/40 of synthesized cDNA in addition to the appropriate oligonucleotide primer pair and run on the ABIPrism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Reverse transcriptase controls were done in parallel without adding enzyme. Obtained cycle numbers were normalized to β-actin. All primer sequences were designed with the Primer Express software (Applied Biosystems). The primer sequences and their final concentrations used were as follows: Axin2 (forward, 600 nM), 5’-GAGGTG GTACCTTGCCAAAA-3’, Axin2 (reverse, 50 nM), 5’-TTCCTGTCCCT CTGCTGACT-3’, Irf1 (forward, 600 nM), 5’-CCCTGGCTAGAGTGCAGGATTAT-3’, Irf1 (reverse, 600 nM), 5’-CATGGATCTGGAATCTGGAAGATCATCTCTT-3’, β-actin (reverse, 40 nM), 5’-GTGGTGTGTTAAGGCATGTAGCAG-3’, β-actin (reverse, 40 nM), 5’-GACGACATGGAGAAGATCTGG-3’.

**Two-dimensional gel electrophoresis of total cell extracts**

Wild-type and Piasy-lacZ homozygous mutant Abelson MuLV-transformed pre-B cell lines were cultured under standard conditions. Total cell extracts were generated in two-dimensional gel sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 100 mM DTT, 0.2% biotin 3-10, (Bio-Rad, Hercules, CA), 10 mM iodoacetamide, 0.5 mM PMSF in the presence of standard protease inhibitors) and the total protein concentration was determined using PlusOne 2-D Quant kit (Axygen Biosciences, Piscataway, NJ). A total of 300 μg of protein was separated in the first dimension using ReadyStrip IGP strip (Bio-Rad), pH 3–10, nonlinear, according to suppliers instructions (Bio-Rad). Focusing proceeded from 0 up to 60 kVh over 9 h followed by 12% SDS-PAGE in the second dimension. After fixation (5% acetic acid, 5% methanol) protein spots were visualized by colloidal Coomassie staining. For anti-SUMO1 immunoblot analysis (anti-GMP-1 mouse monoclonal, 1:1000; Zytomed, Berlin, Germany), two-dimensional gel electrophoresis was performed as described followed by standard blocking and immobilobilizing techniques.

**Results**

**Targeted disruption of the Piasy gene**

With the aim of inactivating the murine Piasy gene in vivo, we used a cloned 10-kb genomic Piasy DNA fragment to generate an insertion-type targeting construct, in which a LacZ-PGKNeo gene cassette has been inserted in-frame into the second exon of the Piasy gene (Fig. 1A). Homologous recombination of the targeting construct has been shown in the parental D3 ES cell line. The targeting vector was microinjected into the zona pellucida of fertilized eggs of C57BL/6J (B6) mice. Genomic DNA was prepared from individual ES cell clones and subjected to Southern blot analysis of EcoRV digested genomic DNA using a 5.3 kb EcoRV fragment containing a 4.7 kb genomic Piasy fragment as the probe. As shown in Figure 1B, four targeted clones (61, 104, 108, and 198) were isolated, and one clone was selected for further analysis. In contrast to the parental D3 ES cell line, homologous recombination in the ES cell clones 61, 104, 108, and 198 was shown by the presence of a 14.9 kb EcoRV fragment containing a 10.3 kb fragment derived from the genomic Piasy locus, while a 14.9 kb EcoRV-PGKNeo fragment is present in the parental D3 ES cell line. These findings confirm homologous recombination of the targeting construct (Fig. 1C). Southern blot analysis of EcoRV digested genomic DNA from ES cell line 61 using different restriction enzymes (EcoRV, HindIII, BamHI, XhoI) confirmed the presence of the targeted construct (Fig. 1D). These findings confirm homologous recombination of the targeting construct. Figure 1E shows a Southern blot analysis of genomic DNA from targeted ES cell clone 61 using EcoRV-PGKNeo and LacZ external probes, confirming homologous recombination of the targeting construct in the ES cell line. The blot was probed with LacZ external probe (5.3 kb external probe) restriction digests of the targeted ES cell clones. The 14 kb and 10.3 kb DNA fragments generated by XhoI/HindIII (5’ external probe), EcoRV (internal probe), and BamHI (3’ external probe) restriction digests of the wild-type and mutant allele are indicated below. Representative restriction sites are indicated above the lines as: B, BamHI; H, HindIII; R, EcoRI; X, XhoI. Wild-type Piasy protein is shown schematically on the top. Piasy contains a putative chromatin-binding SAP (SAP) domain, a C2HC3 RING (RING), a C-terminal serine-rich and acidic (Ser/Ac) domain. The nuclear localization signal (NLS, [ ]) is indicated. Numbers below indicate the amino acid positions of the respective protein domains. The Piasy-β-Gal fusion protein, schematically shown below, retains the SAP domain and the nuclear localization signal, but inactivates the RING and the serine-rich and acidic domains of the Piasy protein. The C and D DNA blot analysis of targeted ES cell clones. The targeting vector was introduced into D3 ES cells by electroporation. The presence of an additional 10.9 kb fragment (5’ external probe (C) and a 10.3 kb fragment (3’ external probe (D) indicates homologous recombination in the ES cell clones 61, 104, 108, and 198 in contrast to the parental D3 ES cell line. E, DNA blot analysis of genomic DNA from representative litters generated by heterozygous matings using the 3’ external probe for hybridization. The 14 kb and 10.3 kb DNA fragments are generated from the wild-type and mutant allele, respectively. F, Immunoblot analysis of total tissue extracts from adult testis obtained from wild-type and Piasy-lacZ homozygous mutant mice. The blot was probed with a monoclonal anti-mouse Piasy Ab. Expression of the Piasy-lacZ allele yields a stable Piasy-β-Gal fusion protein. The positions of the 60 kDa wild-type Piasy and the Piasy-β-Gal fusion protein expressed by homozygous mutant Piasy-lacZ mice are indicated.
construct in murine ES cells should yield cell clones in which amino acid 81 of PIASy has been fused with the N terminus of the bacterial β-Gal protein. This targeting strategy should allow for the visualization of the expression pattern of the PIas gene in heterozygous mutant mice and the inactivation of the gene in homozygous mutant mice because the fusion protein lacks most of the PIASy protein domain, including the functionally important RING domain. Mutations in the RING domain have been found to abrogate the function of PIAS proteins in vitro and in transfection assays (3, 22, 33–35), and therefore we will refer to the homozygous PIas-lacZ mutation also as PIas \textsuperscript{−/−} mutation.

The linearized targeting construct was transfected into D3 ES cells and clones that had been subjected to positive and negative selection were screened for homologous recombination by digesting genomic DNA with EcoRV and hybridizing DNA blots with an internal probe, derived from genomic sequences at the end of intron 2 of the PIas gene (Fig. 1A, data not shown). Ten targeted ES cell clones were identified among 108 analyzed colonies and the homologous recombination events were confirmed by hybridization of XhoI/BamHI- and BamHI-digested genomic DNA with 5′ and 3′ external probes, respectively (Fig. 1, A, C, and D). Six targeted ES cell clones were microinjected into C57BL/6 blastocysts and chimeric males were crossed with C57BL/6 wild-type females. One chimera, derived from ES cell clone 198, transmitted the targeted allele through the germline. The genotypes of the F1 offspring were determined by PCR (data not shown) and DNA blot analysis of BamHI-digested genomic DNA that had been hybridized with a 3′ external probe (Fig. 1E). Heterozygous animals did not show any obvious differences in phenotype or fertility as compared with wild-type littermates (data not shown). However, genotyping of 209 offspring from heterozygous intercrosses revealed a significantly reduced Mendelian frequency of 14%, instead of 25%, homozygous mutant (PIas \textsuperscript{−/−}) mice. The Mendelian frequency of homozygous mutant PIas \textsuperscript{−/−} embryos up to E18.5 was normal, suggesting a decreased perinatal viability. The surviving PIas-deficient mice showed no obvious phenotype and were fertile.

To confirm that the targeted PIas allele does not produce functional PIASy protein (Fig. 1B), we prepared total protein extracts from testis of wild-type and homozygous mutant (PIas \textsuperscript{−/−}) mice and assayed the extracts for the presence of PIASy protein by immunoblot analysis. Using a rat anti-PIASy mAb directed against amino acids 1 to 97 of the mouse PIASy protein, we failed to detect wild-type PIASy protein in the mutant tissue extract, although the slower-migrating PIASy-β-Gal fusion protein was readily detectable (Fig. 1, B and F).

**Analysis of PIas expression during embryonic development and morphology of PIas-deficient mice**

With the aim of visualizing individual cells and tissues that express the PIas gene in the embryo, we performed whole mount β-Gal staining of heterozygous PIas-lacZ embryos at E12.5 and E14.5 (Fig. 2B and data not shown). Abundant expression of PIASy-β-Gal was detected in multiple tissues, including brain, skin, limb buds, tail bud, whisker follicles, and pelage hair follicle primordia (Fig. 2B). In sagittal cryosections of heterozygous PIas-lacZ embryos at E16.5, an almost ubiquitous expression pattern could be observed with stronger expression in the neopallial cortex and the ventricular zone of the brain, the midbrain, the developing cerebellum, throughout the spinal cord and in the olfactory epithelium (Fig. 2E). Strong PIASy expression was also detected in the epidermis, the dorsal surface of the tongue, in the trapezius muscle and the cartilage primordium of the ribs and bones, the thyroid cartilage, tracheas, and lung (Fig. 2E). In wild-type embryos, no β-Gal staining was detected, except the duodenum, indicating the observed expression in heterozygous mice is specific (Fig. 2D). These results correspond to previous in situ hybridization studies of E8.5 up to E16.5 mouse embryos, which detected PIas transcripts in the limbs, the neuroepithelium, and hair follicles (55). Taken together, these studies suggest that PIASy may play a modulatory role during the development of multiple organs.

Morphological analysis of sections of PIas \textsuperscript{−/−} mice at E14.5 and E16.5 did not reveal any obvious abnormality (Fig. 2, C and F). In addition, no altered expression pattern or mislocalization of the PIas-β-Gal fusion protein is detected in PIas-lacZ homozygous mutant mice (Fig. 2F). Although adult PIas \textsuperscript{−/−} mice also show no obvious phenotype, the basis for the reduced viability of these mice remains to be established.

**PIASy expression in hemopoietic cells and analysis of cell populations in PIASy-deficient mice**

To address the question of whether PIASy is specifically expressed in distinct hemopoietic cell populations, we incubated cells of lymphoid organs with fluorescein-digalactoside, a fluorogenic substrate for β-Gal to detect Lac-Z-positive cells by flow cytometry. Using this approach, we found that PIASy is abundantly expressed in developing and mature T cells of the thymus (Fig. 3A). Histogram overlays of cells gated for PIASy-β-Gal expression indicated that 98–99% of CD4\textsuperscript{+}CD8\textsuperscript{−} double positive cells, CD4\textsuperscript{−} single positive and CD8\textsuperscript{−} single positive cells express PIASy at abundant levels (Fig. 3A). Analysis of the T cell compartments in thymus and spleen of wild-type mice and PIas-lacZ homozygous mutant
mice by flow cytometry indicated that the numbers of CD4\(^+\) CD8\(^+\) double positive cells, as well as the numbers of CD4\(^+\) and CD8\(^+\) single positive cells, are equivalent in both wild-type and mutant mice (Fig. 3, B and C).

B220\(^+\) CD43\(^+\) pre-B cells of the bone marrow and mature B cells of the spleen (B220\(^+\) IgM\(^-\)) were found to express high levels of PIASy-\(\beta\)-Gal (Fig. 3, D and F). However, the analysis of the B cell compartments in bone marrow and spleen of homozygous mutant mice showed a normal B220/CD43 profile and B220/IgM profile, respectively (Fig. 3, E and G).

Finally, PIASy-\(\beta\)-Gal expression was detected at lower levels in myeloid cells of bone marrow, using the markers Mac1 and GR1 (Fig. 3H). Analysis of the myeloid compartment in the bone marrow of PIasy\(^{-/-}\) mice showed modest but reproducible 20\% increase in the numbers of Mac1\(^+\) GR1\(^+\) double positive cells (Fig. 3I). Thus, PIASy is abundantly expressed in multiple cell types of the lymphoid and myeloid lineages. However, PIASy is not essential for normal B and T cell development and has a modest effect on myeloid cell differentiation.

**Cytokine signaling in PIasy-lacZ homozygous mutant mice**

IFN-\(\alpha\) and IFN-\(\gamma\) signal to the nucleus by activating the latent cytoplasmic transcription factor STAT1, which is translocated to the nucleus and activates IFN-responsive target genes (reviewed in Refs. 6 and 7). PIASy was shown to act as a transcriptional corepressor of STAT1 and the association of these proteins was found to be augmented by IFN signals (27). This observation has been interpreted to suggest that PIASy plays a role in the attenuation of the IFN response. STAT1 is indispensable for the IFN pathways (56, 57) and therefore, we examined the activity of IFN-\(\alpha\) and IFN-\(\gamma\) to induce target gene transcription in splenocytes and bone marrow-derived macrophages from wild-type or PIasy-\(\alpha\)-lacZ homozygous mutant mice. Using quantitative real-time PCR analysis to detect transcripts of Irf1 gene, a direct target of STAT1 (58, 59), gene expression was induced 5- to 9-fold in both similar to those of wild-type mice (Fig. 4), suggesting that the absence of PIASy does not result in any significant differences between wild-type and PIasy-lacZ homozygous mutant cells (Fig. 5A). Stimulation of the MEFS cells derived with LiCl resulted in quite variable induction efficiencies, ranging from 5.6-fold (Piasy\(^{-/-}\) MEF 17) up to 24.2-fold (Piasy\(^{-/-}\) MEF 12) (Fig. 5A). The induction efficiencies of the wild-type MEF cell lines ranged between 7.8-fold (wild-type MEF 5) and 13.1-fold (wild-type MEF no. 18). Therefore, the Wnt response of Axin2 in PIASy-deficient MEFS is comparable to that in wild-type MEFS.

To further assess the Wnt responsiveness of wild-type and PIasy\(^{-/-}\) MEFS, we transfected transiently these cells with a LEF1 reporter construct, in which multimerized binding sites for LEF1/TCF proteins are linked to the minimal c-fos promoter and luciferase gene (LEF-\(\gamma\)-fos-luciferase). Previously, we found that PIASy represses the LEF1- and \(\beta\)-catenin-dependent activation of this reporter construct in transiently transfected 293 cells (3). In a first experiment, we compared the expression levels of the transfected LEF1 reporter in wild-type with PIASy-deficient MEFS cells in the absence or presence of the surrogate Wnt signal, LiCl, but without the addition of exogenous LEF1 or \(\beta\)-catenin expression constructs (Fig. 5B). The basal levels of reporter activity were similar in NaCl-treated wild-type and mutant MEFS cells (Fig. 5B). LiCl treatment of these cells induced a 2- to 3-fold increase in reporter gene expression in both wild-type and mutant MEFSs, suggesting that the loss of functional PIASy had no effect on basal or Wnt-induced reporter gene activity. Because the effects of LiCl in MEFSs were limited, possibly due to the presence of low levels of endogenous LEF1/TCF proteins, we compared the activity of the LEF-\(\gamma\)-fos-luciferase reporter in wild-type and PIasy-\(\alpha\)-lacZ homozygous mutant MEFSs in the presence of exogenous LEF1/TCF proteins. We examined the activity of the LEF-\(\gamma\)-fos-luciferase reporter in wild-type and PIasy-\(\alpha\)-lacZ homozygous mutant MEFSs in the presence of exogenous LEF1 and \(\beta\)-catenin. Cotransfection of LEF1 and \(\beta\)-catenin expression plasmids into wild-type MEFSs resulted in a robust 11.2-fold activation of the LEF-\(\gamma\)-fos-luciferase reporter (Fig. 5C, wt MEF 18). However, in PIasy\(^{-/-}\) MEFSs, the transcriptional activation of the reporter construct was reduced almost 3-fold (Fig. 5C, MEF 11), whereas no effect was observed in cells that have been transfected with only LEF1 or \(\beta\)-catenin expression plasmids. This result suggests that PIASy may modulate the Wnt responsiveness of genes in vivo.

The modest effects of the deficiency of PIASy could be, in principle, accounted for by a redundancy with another member of the PIAS family and/or an up-regulation of the expression of another PIAS gene. Therefore, we examined the expression of the PIAS genes in wild-type and PIasy\(^{-/-}\) MEFSs. To this end, we performed a quantitative real-time PCR analysis to detect transcripts of the Pias1, Pias3, and Piasx genes. The levels of expression of these Pias genes were found to be similar in wild-type and PIASy-deficient MEFSs, suggesting that the absence of PIASy does not result in a compensatory up-regulation of the expression of another Pias gene (data not shown).

**Wnt signaling in PIasy-lacZ homozygous mutant mice**

LEF1/TCF proteins are nuclear mediators of Wnt signals that activate transcription of Wnt-responsive target genes in conjunction with \(\beta\)-catenin (reviewed in Ref. 10). LEF1 and TCF4 were found to interact specifically with PIASy, whereby the activity of LEF1 is repressed and the activity of TCF4 is enhanced (3, 4). Biochemical and genetic analyses identified Axin2, which acts as a negative regulator of soluble \(\beta\)-catenin, as a target of Wnt signaling, thus establishing a negative feedback loop for Wnt signaling (60). To examine the Wnt responsiveness of the endogenous Axin2 gene in wild-type and PIasy-lacZ homozygous mutant mice, we deter-

mined the levels of Axin2 mRNA in MEFS cell lines that have been treated with LiCl, which inhibits glycogen-synthase-kinase-3\(\beta\) and acts as a surrogate Wnt signal (61). For this experiment, we used two wild-type and four mutant mice to derive primary MEFSs, and we performed the expression analysis with duplicate samples in three independent experiments. Determination of basal Axin2 levels in untreated MEFSs by quantitative real-time PCR analysis did not reveal any significant differences between wild-type and PIasy-\(\alpha\)-lacZ homozygous mutant cells (Fig. 5A). Stimulation of the MEFS cells derived with LiCl resulted in quite variable induction efficiencies, ranging from 5.6-fold (Piasy\(^{-/-}\) MEF 17) up to 24.2-fold (Piasy\(^{-/-}\) MEF 12) (Fig. 5A). The induction efficiencies of the wild-type MEF cell lines ranged between 7.8-fold (wild-type MEF 5) and 13.1-fold (wild-type MEF no. 18). Therefore, the Wnt response of Axin2 in PIASy-deficient MEFSs is comparable to that in wild-type MEFSs.

To further assess the Wnt responsiveness of wild-type and PIasy\(^{-/-}\) MEFSs, we transfected transiently these cells with a LEF1 reporter construct, in which multimerized binding sites for LEF1/TCF proteins are linked to the minimal c-fos promoter and luciferase gene (LEF-\(\gamma\)-fos-luciferase). Previously, we found that PIASy represses the LEF1- and \(\beta\)-catenin-dependent activation of this reporter construct in transiently transfected 293 cells (3). In a first experiment, we compared the expression levels of the transfected LEF1 reporter in wild-type with PIASy-deficient MEFS cells in the absence or presence of the surrogate Wnt signal, LiCl, but without the addition of exogenous LEF1 or \(\beta\)-catenin expression constructs (Fig. 5B). The basal levels of reporter activity were similar in NaCl-treated wild-type and mutant MEFS cells (Fig. 5B). LiCl treatment of these cells induced a 2- to 3-fold increase in reporter gene expression in both wild-type and mutant MEFSs, suggesting that the loss of functional PIASy had no effect on basal or Wnt-induced reporter gene activity. Because the effects of LiCl in MEFSs were limited, possibly due to the presence of low levels of endogenous LEF1/TCF proteins, we compared the activity of the LEF-\(\gamma\)-fos-luciferase reporter in wild-type and PIasy-\(\alpha\)-lacZ homozygous mutant MEFSs in the presence of exogenous LEF1 and \(\beta\)-catenin. Cotransfection of LEF1 and \(\beta\)-catenin expression plasmids into wild-type MEFSs resulted in a robust 11.2-fold activation of the LEF-\(\gamma\)-fos-luciferase reporter (Fig. 5C, wt MEF 18). However, in PIasy\(^{-/-}\) MEFSs, the transcriptional activation of the reporter construct was reduced almost 3-fold (Fig. 5C, MEF 11), whereas no effect was observed in cells that have been transfected with only LEF1 or \(\beta\)-catenin expression plasmids. This result suggests that PIASy may modulate the Wnt responsiveness of genes in vivo.

The modest effects of the deficiency of PIASy could be, in principle, accounted for by a redundancy with another member of the PIAS family and/or an up-regulation of the expression of another PIAS gene. Therefore, we examined the expression of the PIAS genes in wild-type and PIasy\(^{-/-}\) MEFSs. To this end, we performed a quantitative real-time PCR analysis to detect transcripts of the Pias1, Pias3, and Piasx genes. The levels of expression of these Pias genes were found to be similar in wild-type and PIASy-deficient MEFSs, suggesting that the absence of PIASy does not result in a compensatory up-regulation of the expression of another Pias gene (data not shown).

**Two-dimensional gel electrophoresis of wild-type and PIasy\(^{-/-}\) cell extracts**

To investigate whether PIASy modulates the expression of Wnt or STAT target genes and to assess whether a deficiency of PIASy alters the SUMO modification of proteins, we adopted a protein expression profiling approach. Total cell extracts of wild-type and PIasy\(^{-/-}\) Abelson-MuLV-transformed pre-B cells were separated
FIGURE 3. PIASy expression analysis in hemopoietic cells and phenotypic characterization of the hemopoietic system of Piasy-lacZ homozygous mutant mice. The expression of PIASy was determined in heterozygous Piasy-lacZ mice using fluorescein-digalactoside (FDG), a fluorogenic substrate for β-galactosidase (β-Gal). A, PIASy expression in the thymus. Thymocytes from heterozygous Piasy-lacZ mice were stained for the expression of CD4, CD8, and PIASy-β-Gal. The representative CD4/CD8 profile indicating the selected gates and quadrant statistics is shown on the left. Right, overlay histograms of PIASy-β-Gal expression in CD4+CD8−, CD4−, and CD8+ cells indicate that PIASy is expressed in all three compartments. B and C, CD4/CD8 profile of thymic (B) and splenic (C) T cells from wild-type (+/+) and Piasy-lacZ homozygous mutant mice. The T cell compartment in Piasy-lacZ homozygous mutant mice is normal. D and F, PIASy expression analysis in the B cell compartment of bone marrow (D) and spleen (F). Total bone marrow cells (D) and splenocytes (F) from heterozygous Piasy-lacZ mice were stained for the expression of B220, CD43, IgM, and PIASy-β-Gal. D, The B220+CD43+ with the selected gate for the PIASy expression analysis in bone marrow (6.1% of living cells) is shown on the left. Right, the overlay histogram of PIASy-β-Gal expression in the B220+CD43+ double positive cell population indicates decent PIASy expression in developing B cells. F, Overlay histograms of PIASy-β-Gal expression of B220+ and IgM+ single positive B cells. The percentage of cells gated for the PIASy-β-Gal expression analysis is indicated in brackets. PIASy is expressed in splenic B cells. E and G, Analysis of the B cell compartment of bone marrow (E) (Figure legend continues)
in two dimensions by isoelectric focusing followed by SDS-PAGE. Protein spots were visualized by colloidal Coomassie staining and the gels were scanned for PDQUEST (Bio-Rad) software analysis (Fig. 6, A and B). We prepared pre-B cell extracts from two wild-type and two PIASy-deficient mice and assayed the extracts in duplicates by two-dimensional electrophoresis (Fig. 6, A and B). The comparison of the pattern of Coomassie-stained protein spots in wild-type and mutant cell extracts by the PDQUEST software (Bio-Rad) showed no differential expression pattern of proteins or shifts in protein migration due to potential secondary modifications of proteins comparing total cell extracts from wild-type (Fig. 6A) and PIasy-lacZ homozygous mutant Abelson MuLV-transformed pre-B cells (Fig. 6B).

To identify potential protein substrates for SUMO modification by PIASy, total protein extracts of wild-type and PIasy-lacZ homozygous mutant Abelson MuLV-transformed pre-B cells were separated by two-dimensional gel electrophoresis and transferred to nitrocellulose membranes for immunoblot analysis using a mouse monoclonal anti-SUMO1 Ab (anti-GMP-1; Zytomem). No significant difference in the pattern of immunoreactive spots could be detected comparing the anti-SUMO1 immunoblots from wild-type and PIasy-lacZ homozygous mutant Abelson MuLV-transformed pre-B cells (Fig. 6, C and D).

Discussion

To date, the functional analysis of mammalian PIAS proteins, which identified these proteins both as regulators of nuclear mediators of various signaling pathways and as SUMO E3 ligases, involved transient transfections and overexpression of both PIAS and partner proteins in cell lines. With the aim of determining the biological role of PIAS proteins in a more physiological context, we undertook the genetic inactivation of a particular member of the PIAS family, PIASy, and examined the effects of the mutation in primary cells. This analysis revealed a modest reduction of the transcriptional response of IFN-γ in wild-type mice and found that the activation potential of a Wnt-responsive reporter by LEF1 and β-catenin is decreased. Together, these data suggest that PIASy may play a regulatory role in signaling by IFN-γ and Wnt proteins in vivo.

The subtle effects of the PIasy-lacZ mutation raise the question of whether the insertion of the lacZ gene into the second exon of PIasy generates a null allele or whether homozygous PIasy-lacZ mice retain a residual activity of PIASy protein. The immunoblot analysis of testis tissue extracts from mutant mice indicates that neither wild-type PIASy nor smaller forms of PIASy protein can be detected. This observation rules out the principle possibility that exon skipping generates a PIASy protein, lacking only the exon with the lacZ insertion. Although the N-terminal SAP domain is retained in the PIASy-β-Gal fusion protein, this protein lacks the central RING domain, which contributes to the interaction with partner proteins and is essential for the functional activity of PIASy. Mutations in the RING domain of PIASy and other PIAS proteins have been shown to abrogate the ability of these proteins to modulate the activity of partner proteins and act as SUMO E3 ligases (3, 22, 34, 35).

Several lines of evidence suggest that other PIAS proteins may compensate, at least in part, for the deficiency of PIASy in the mouse. First, PIAS proteins share a similar domain structure and show overlapping specificities in the association and sumoylation of partner proteins. For example, transient overexpression of PIASy and PIAS1 both result in the repression of a STAT1-responsive reporter construct in transfection experiments (2, 27).

and spleen (G) in wild-type and PIasy-lacZ homozygous mutant mice using the cell surface markers B220, CD43 for bone marrow (E), and B220, IgM for spleen (G). No abnormalities can be observed in the B cell compartment of PIasy-lacZ homozygous mutant mice. H, PIASy expression in the myeloid lineage. Total bone marrow cells from heterozygous PIasy-lacZ mice were stained for the expression of Mac1, GR1, and PIASy-β-Gal. The Mac1/GR1 profile with the selected gate for the PIASy expression analysis (42.4% of the living cell population) is shown on the left. Right, the overlay histogram of PIASy-β-Gal expression in Mac1/GR1 double positive cells indicates that PIASy is expressed. I, Mac1 and GR1 expression analysis in bone marrow of wild-type and PIasy-lacZ homozygous mutant mice. No major abnormality can be observed in myeloid cells of PIasy-lacZ homozygous mutant mice.
Moreover, PIAS1 and PIASx proteins both act as SUMO E3 ligases and corepressors of AR and p53 (12, 22, 34, 35). However, some specificity of PIAS proteins can be demonstrated in transfection assays. PIASxα, but not PIAS3, antagonizes the IL-12-dependent activation of a reporter gene by STAT4 (5). Likewise, the repression and sumoylation of the nuclear matrix protein SATB2 is mediated specifically by PIAS1, but not by PIASx and PIAS3 (62). Second, PIAS proteins are widely expressed and show overlapping pattern of expression in the developing mouse (55). Our analysis of heterozygous and homozygous Piasy-lacZ embryos shows an almost ubiquitous expression at a low level but a more specific expression in multiple tissues. Third, a redundancy of PIAS proteins has been found in yeast, in which two PIAS-related proteins, termed Siz1 and Siz2, act redundantly in promoting SUMO modification of septins (14). Both Siz1Δ and Siz2Δ strains show no obvious phenotypes, but the double mutation resulted in poor cell growth and accumulation in G2/M of the cell cycle. Moreover, the overall sumoylation of proteins was found to be diminished in Siz1Δ cells but was virtually abrogated in Siz1ΔSiz2Δ cells (14).

One obvious effect of the Piasy-lacZ mutation was the reduced viability of newborn mice. In contrast to the normal Mendelian frequency of homozygous mutant mice before birth, we observed only 14% homozygous mutants at weaning age. As we could not detect any obvious abnormalities, the basis for the reduction of the viability is unknown. The reduced viability may also reflect some heterogeneity in the genetic background of the mice, which were generated in a 129 strain but were backcrossed to a C57BL/6 strain. Strain-specific variations in the penetrance of mutations have been observed (63).

Although PIASy is expressed in multiple hemopoietic cell lineages, the only effect of the Piasy-lacZ mutation that we observed was a 25% increase in the number of Mac-1- and Gr-1-positive myeloid cells in the bone marrow. This modest increase in myeloid cells is reproducible in multiple mice and is consistent with the finding that the expression of Pias3 is lost in leukemic cells in which STAT3 is persistently activated (64, 65). Although PIASy has not been implicated in the regulation of STAT3, it is possible that PIASy and PIAS3 act redundantly in myeloid cells to modulate cytokine signaling.

In macrophages of the bone marrow and to a lesser extent in splenocytes, we detected a reproducible 2- to 3-fold decrease in the IFN-γ responsiveness of the endogenous Irf1 gene, a member of the IRF family of transcription factors, that is a direct target gene of IFN signaling (58, 59). Irf1 is involved in the regulation of the expression of both IFN-stimulated genes and IFN genes themselves. The induction of Irf1 transcription by IFN-γ in wild-type macrophages appears to be governed specifically by STAT1 because no Irf1 induction is observed in IFN-γ-treated macrophages in which STAT3 is persistently activated (64, 65). Although PIASy has not been implicated in the regulation of STAT3, it is possible that PIASy and PIAS3 act redundantly in myeloid cells to modulate cytokine signaling.

In macrophages, the asymmetric ATF2-c-Jun site impairs its virus induction (68). For example, replacement of the ATF2-c-Jun site with an AP-1 site in the IFN-β promoter, or reversal of the orientation of the asymmetric ATF2-c-Jun site impairs its virus induction (68). Therefore, changes in the context of transcription factor binding sites can result in different regulatory contributions of proteins. Second, we examined the effects of PIASy in primary cells and

FIGURE 5. Analysis of Wnt responsiveness in wild-type vs Piasy-lacZ homozygous mutant MEFs. A, Axin2 mRNA levels were determined by real-time PCR analysis of wild-type and Piasy-lacZ homozygous mutant MEFs after stimulation with 30 mM LiCl (as a surrogate Wnt signal, (Ref. 61) and 30 mM NaCl (control) for 8 h. Axin2 mRNA levels were normalized to β-actin and the fold-change was calculated by setting the normalized relative Axin2 level of the unstimulated (30 mM NaCl treatment) wild-type control MEF cell line no. 5 to one. Basal Axin2 levels (unstimulated, 30 mM NaCl, □) do not differ between wild-type and mutant MEFs. Numbers above the bars indicate the fold-change of the Axin2 mRNA levels comparing each unstimulated (30 mM NaCl, □) with stimulated (30 mM LiCl, ■) MEF cell line of the indicated genotype. The data were derived from three independent experiments performed in duplicates. B, Wnt-responsiveness of the LEF-1-luciferase reporter (1 μg) was examined in transient transfection assays of wild-type vs mutant MEFs by treatment of transfected cell pools with 30 mM LiCl as a surrogate Wnt stimulus and 30 mM NaCl as control, respectively. Numbers given indicate the fold-changes in reporter gene activity in response to LiCl relative to the control NaCl treatment, which is set to one. C, LEF1-dependent reporter gene activity was examined by transient transfection assays of wild-type and mutant MEFs using 1 μg of LEF1-luciferase reporter in the absence or presence of 50 ng of LEF1 and 0.5 μg of β-catenin. Data shown are duplicates of a representative transfection.

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without the overexpression of proteins, which occurs typically in transfection assays and may squelch a regulatory response by titrating regulatory cofactors. Although the relatively modest effect of the PIASy deficiency on Irf1 expression may reflect a redundancy of PIASy with other PIAS proteins, such as PIAS1, we cannot rule out the possibility that the contribution of PIAS proteins to the regulation of signaling by STAT proteins is limited. The overexpression of PIASx/H9251 augments the activation of a synthetic reporter construct by STAT1 in IFN-H9253-treated U3a cells, which lack endogenous STAT1 only 2-fold (36).

In contrast to the modest reduction of the IFN-H9253 response in PIASy-deficient macrophages and splenocytes, no changes could be observed in the IFN-H9251 response. Both IFNs use STAT1 as a nuclear mediator but the responses differ in the specificity of transcription factor activation. IFN-γ activates STAT1 as a dedicated transcription factor, whereas IFN-α activates both STAT1 and STAT2 (69, 70). In addition, other signaling molecules have been found to activate STAT1 in vitro. These have been shown to include epidermal growth factor, M-CSF, IL-6, IL-10, and others (71–74). STAT1-deficient mice show a normal responsiveness to these other cytokines, whereas both IFN-α- and IFN-γ-signaling was impaired, indicating that STAT1 is a transcription factor dedicated to IFN signaling (56, 57, 75). The basis for the differential effect of PIASy on signaling by IFN-α and IFN-γ is unclear but it may be related to the cross-talk between these signaling pathways (6).

Based on the association of LEF1/TCF proteins with PIASy in vitro, we also anticipated an alteration in the expression of a Wnt-responsive reporter construct by LEF1 and β-catenin in PIASy-deficient MEFs. We have previously shown that PIASy represses the activity of LEF1 in transiently transfected HeLa or 293 cells (3). In addition, PIASy was found to augment the activation of a LEF1/TCF reporter by TCF4 and β-catenin (4), which is consistent with our observation that the activation potential of LEF1 in PIASy-deficient MEFs is reduced relative to that in wild-type MEFs. In our previous transfection experiments, in which we observed repression of the LEF1 activity by PIASy, we have used 293 and HeLa cells and noted that the repression was not observed in fibroblastic cells, suggesting that the regulation of transcription factor activity by PIASy is cell type-specific (3). In contrast to the

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**FIGURE 6.** Two-dimensional gel electrophoresis analysis of wild-type and Piasy-lacZ homozygous mutant pre-B cell lines. Total protein extracts were generated from MuLV transformed pre-B cell lines derived from wild-type (+/+) and Piasy-lacZ homozygous mutant embryos. A total of 300 μg of total protein were separated by isoelectric focusing in the first dimension using a nonlinear gradient of pH 3–10, followed by 12% PAGE in the second dimension. A and B, Colloidal Coomassie staining of two-dimensional gels performed from wild-type (A) and Piasy-lacZ homozygous mutant (B) total protein extracts. No major differences in the overall staining pattern of wild-type and mutant extracts could be observed. C and D, Anti-SUMO1 immunoblot analysis of two-dimensional gels performed from wild-type (C) and Piasy-lacZ homozygous mutant (D) total protein extracts. No significant differences in the immunoreactive patterns of wild-type and Piasy-lacZ homozygous mutant protein extracts could be observed.
changes of LEF1- and β-catenin-mediated activation of a reporter construct in PIASy-deficient MEFs, we did not detect any significant change in the Wnt-response of the endogenous Axin2 gene, a known Wnt target gene (60). In MEFs, the amount of LEF/TCF proteins is possibly too low to elicit a strong Wnt response and PIASy may have an effect only under conditions of strong Wnt signals.

A common hallmark of PIAS proteins is their ability to modulate the transcription of proteins with SUMO (38). Therefore, we examined whether the sumoylation pattern of proteins is altered in PIASy-deficient cells. In transformed pre-B cells from mutant mice, we did not detect a significant change in the pattern of protein expression relative to wild-type mice. Notably, the sumoylation of proteins by PIAS proteins does not necessarily correlate with changes in their activity. For example, PIAS1 and PIASx both modulate the activity of STAT1 in transfection assays (36, 40). However, the sumoylation of STAT1 by PIASx does not correlate with changes in transcriptional activation as a mutation of the SUMO acceptor site at lysine 703 of STAT1 has no effect on STAT1 activity (36). Likewise, mutations of two SUMO acceptor sites of LEF1 have no effect on the activity of LEF1, although the association with PIASy and the presence of an intact RING domain is required for the repression of LEF1 in 293 cells (3). In contrast, sumoylation of some proteins, such as AR and SATB2 alters their activities (35, 62). Thus, PIAS proteins may use multiple mechanisms to modulate the activities of partner proteins.

In conclusion, the analysis of the PIASy-deficient mice has revealed a functional role for PIASy in the viability of mice and IFN-γ signaling, although the effects are modest. Therefore, it will be of interest to examine the effects of mutations of multiple PIAS genes in the mouse to determine the role of PIAS proteins in sumoylation and regulation of transcription factor activity. While this study was under review, an independent study analyzing the effects of targeted mutations of similar conclusions (76).

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References


