Gfi1: Green Fluorescent Protein Knock-in Mutant Reveals Differential Expression and Autoregulation of the Growth Factor Independence 1 (Gfi1) Gene during Lymphocyte Development*

Raif Yücel, Christian Kosan, Florian Heyd, and Tarik Mörüy‡

From the Institut für Zellbiologie (Tumorforschung), Universitätsklinikum Essen, Virchowstraße 173, D-45122 Essen, Germany

The Gfi1 gene encodes a 55-kDa transcriptional repressor protein with important functions in T-cell development, in granulopoiesis, and in the regulation of the innate immune response. To follow expression of the Gfi1 gene during the differentiation of specific immune cells, we have generated a mouse mutant in which the Gfi1 coding region is replaced by the gene for the green fluorescent protein (GFP). We found that Gfi1 gene expression is highest in early B-cell subpopulation and differentially expressed during T-cell development with peak levels at stages where pre-TCR or positive/negative selection takes place. Gfi1 is absent in mature B-cells, whereas in peripheral T-cells Gfi1 gene expression is low but rises significantly upon T-cell receptor triggering and decreases again in T-memory cells. Constitutive expression of an lck promoter-driven Gfi1 transgene led to transcriptional silencing of the Gfi1:GFP allele in T-cells. Because Gfi1 was found to occupy genomic sites of its own promoter in thymocytes and was able to repress its own transcription in vitro we propose that transcription of the Gfi1 gene is regulated through an autoregulatory feedback loop.

The Gfi1 gene was first discovered during analysis of proviral integration sites and their associated target genes in the NB2 rat lymphoma cell line after retroviral infection with the non-acute transforming Moloney murine leukemia virus (MoMuLV) (1). In similar experiments, the Gfi1 gene was found to be transcriptionally activated by proviral insertion in T-lymphoid tumors that arose in normal and tumor-prone transgenic mice after infection with MoMuLV (2–4). These findings suggested a function of Gfi1 as a dominant proto-oncogene, which was supported by studies with transgenic mice constitutively overexpressing Gfi1 in T-cells (5). The Gfi1 gene encodes a 55-kDa nuclear protein that harbors six C-terminal C2-H2 zinc finger domains and a characteristic N-terminal 20-amino acid stretch that was termed “SNAG” domain, because it is well conserved between Gfi1 and the proteins Snail and Slug, which also bear similar zinc finger domains (6). Gfi1 binds to specific DNA sequences, and this ability depends on the presence of some but not all of its zinc finger domains. Experiments with reporter genes driven by synthetic promoters with upstream Gfi1 binding sites suggested a transcriptional repressor activity of Gfi1, and further mutational studies clearly delineated that this activity depends on the DNA-binding activity of Gfi1 and on an intact N-terminal SNAG domain (6, 7).

Protein and RNA analyses showed that Gfi1 is expressed during T-cell development beginning at early stages of pre-T-cell selection until the point where T-cells express both CD4 and CD8 surface markers (8, 9). In more mature, CD4 or CD8 single positive (SP) T-cells, Gfi1 is not readily detected but can be induced by stimulation of the T-cell receptor (TCR) (8). Whether the earliest CD4 or CD8 (DN) precursor cells that enter the thymus from the bone marrow and bear the surface markers CD44 (termed DN1 cells) or CD44 and CD25 (termed DN2 cells) express Gfi1 is presently not known. When DN2 cells enter the cell cycle and start to rearrange their TCRβ, γ, and δ chain genes (10, 11), CD44 expression decreases and DN2 cells differentiate into the CD25+/CD44− subset. These “DN3” cells express Gfi1 (9), are strongly proliferating, and can be considered to represent the main source of cells populating the entire thymus. DN3 cells undergo pre-TCR selection (also termed “β-selection”) (12, 13); a process in which only those DN3 cells survive that have productively rearranged their TCR β-chain gene segments; only DN3 cells are eliminated by apoptosis. How Gfi1 expression is regulated during these very early steps of T-cell differentiation remains to be elucidated.

Selected DN3 cells undergo a number of cell divisions before they rearrange and express the TCRα gene locus. Subsequently, DN3 cells down-regulate CD25 and become CD25−/CD44− (DN4) cells and soon start to express the surface markers and co-receptors CD4 and CD8 giving rise to the major DP T-cell population, which constitute over 80% of all cells in the thymus (10, 11). Independently of MHC class type, TCR signaling leads first to a loss or a reduction of co-receptor expression and generates a CD4+CD8+ subpopulation. These cells are thought to be subject to positive/negative selection that ensures the elimination of self-reactive T-cells and the propagation of self-tolerant T-cells (14–16).

Studies with Gfi1-deficient mice or lck-Gfi1 transgenic animals that overexpress Gfi1 constitutively in T-cells suggested that Gfi1 is involved in the cytokine-dependent progression of

* This work was supported by the Deutsche Forschungsgemeinschaft (Grant Mo435/10-4, 10-5), the Fonds der chemischen Industrie, the European Community Framework 5 Program, and the IFORES Program of the University of Essen Medical School. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Institut für Zellbiologie (Tumorforschung), IFZ, Virchowstrasse 173, D-45122 Essen, Germany. Tel.: 49-201-723-3380; Fax: 49-201-723-5904; E-mail: moeroey@uni-essen.de.

† The abbreviations used are: MoMuLV, moloney murine leukemia virus; MHC, major histocompatibility complex; MFU, mean fluorescence intensity; TCR, T-cell receptor; DN, double negative; DP, double positive; SP, single positive; Lin, lineage; GFP, green fluorescent protein; PBS, phosphate-buffered saline; LPS, lipopolysaccharide; wt, wild type; IP, immunoprecipitation; CMV, cytomegalovirus; RT, reverse transcription; FACS, fluorescence-activated cell sorting; LC, large cycling cells; SR, small resting cells.
DN1 cells to the DN2 stage and that Gfi1 plays a role in the pre-TCR-mediated progression of cells through the DN3 stage (9, 17–19). In addition, Gfi1 knock-out mice showed a bias in the CD4/CD8 lineage decision and in the process of positive selection toward a preferred development of CD8+ cells (17). A result of these numerous defects in T-cell development, Gfi1-deficient mice show a severe reduction of thymic cellularity (17). Similar to the development of T-cells, mature B-cells are generated from precursor cells through a series of developmental steps (18). In the earlier phases of B-cell development, precursor cells require cytokines and the appropriate stromal environment, but at later stages more mature cells undergo selection steps that are governed by a pre-B-cell receptor consisting of a μ chain and the surrogate light chains λ5 and V pre-B or the mature BCR complex made up by immunoglobulin heavy and light chains and associated membrane proteins (18). The different stages of B-cell development can be defined by expression of the surface markers c-kit, B220, CD43, and CD25. Expression of Gfi1 has been documented in bone marrow, where B-cell development takes place but a cell type-specific or developmentally regulated expression of Gfi1 in this compartment has not been elucidated yet.

The transcriptional regulation of the Gfi1 gene during T- or B-cell development is presently unknown. To investigate this in more detail, we generated a mouse “knock-in” mutant by gene targeting that carries the coding region of green fluorescent protein (GFP) in the Gfi1 locus. By following the intensity of green fluorescence we were able to monitor Gfi1 gene expression during lymphocyte development and to gain more insight into the regulatory mechanisms of transcriptional control at the Gfi1 locus.

Experimental Procedures

Generation of Gfi1::GFP Knock-in Mice—Gfi1<sup>CProx-cre</sup> mice were generated by homologous recombination in R1 ES cells using procedures that have been described previously. Transfection of ES cells and selection of clones was performed essentially as described before (20). Mice were housed at the animal facility of the Institut für Zellbiologie, University of Essen Medical School in single ventilated cages under specific pathogen-free conditions according to German animal legislation. Mice that were used for analyses were healthy 4- to 6-week-old animals from a backcross of several generations with C57BL/6 animals.

Flow Cytometry Analysis—Single cell suspensions were prepared as described (9) at the time of autopsy from thymus, spleen, lymph nodes, or bone marrow in PBS supplemented with 1% fetal calf serum (staining solution). Cell numbers were calculated using a CASY-1 cell counter (Scharfe System), were washed in this solution, and incubated at 4 °C for 20 min with antibodies that were biotinylated or directly conjugated with fluorochromes. All data were collected on a FACSCalibur (BD Biosciences) and were analyzed with CellQuest (BD Biosciences) and FlowJo (TreeStar) software. To analyze the thymic DN population, antibodies against CD3, CD4, CD8, B220, Gr-1, Mac-1, Ter-119, and DX5, followed by Streptavidin-PerCP-Cy5.5, was used. FlowJo was used for data analysis. Data were gated on the basis of isotype control staining, the mean fluorescence intensity was determined, and the percentage of the total cell population was calculated.

PCR analysis of gene expression—Total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. The amount of RNA was confirmed by spectrophotometry. RT-PCR was performed using Superscript II (Invitrogen) followed by the manufacturer’s protocol. Quantitative RT-PCR was performed in a 20-μl reaction volume containing 900 nM of each primer, 250 μM TaqMan probe, and 1× TaqMan Universal PCR Master Mix (ABI, Germany) according to the manufacturer’s instructions.
manufacturer’s instructions. Reactions were monitored in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). For Gfi1 (Mm00515853_m1) and glyceraldehyde-3-phosphate dehydrogenase (Mm99999915_s1) assays on Demand (ABI, Germany) were used. To amplify GFP mRNA, specific primers were designed using the Assays by Design FileBuilder software (Applied Biosystems) (forward: GGACGGACCGA3 ACTACAAGA; reverse: TCAGCTGATGGGCT TGTCAC; probe: AACCTGGAAGGGCAACCC). To correct for the amount of cDNA added to any individual reaction, PCR was performed in duplicates. The expression of the gene of interest was calculated relative to the glyceraldehyde-3-phosphate dehydrogenase mRNA and was expressed as a Δct values. A correction factor was calculated by comparing Gfi1 and GFP standard curves with different numbers of thymocytes and was used in the final calculation of relative mRNA levels of Gfi1 and GFP genes.

RESULTS

Generation and Functional Testing of the Gfi1:GFP Knock-in Mouse—We have used homologous recombination to generate a reporter mouse line in which parts of the Gfi1 coding region were replaced by a CDN encoding green fluorescent protein (GFP). The GFP gene was inserted in-frame with the ATG translation initiation codon of Gfi1, thereby placing it under the transcriptional control of the Gfi1 regulatory elements. The targeting construct was designed to replace exons 3–5 of Gfi1 by GFP and included a selectable marker gene (Neo) flanked by loxP sites (Fig. 1a). Heterozygous Gfi1<sup>GFP</sup>/Gfp<sup>+</sup> knock-in mice were mated with CMV-Cre transgenic mice to remove theNeo cassette. The Southern hybridization (Fig. 1b) was performed in experiments with previously described mice that were heterozygous for the Gfi1 allele (20). Gfi1<sup>GFP</sup>/GFP<sup>+</sup> mice were indistinguishable from wt mice and were normal with respect to T- and B-cell development when compared with Gfi1 protein level (not shown). In contrast, homozygous Gfi1<sup>GFP/GFP</sup> mice showed the typical phenotype of Gfi1-deficient animals that was described recently (17, 20, 22), including neutropenia, low thymocyte cellularity, and developmental defects during T-cell differentiation (not shown). GFP fluorescence was readily detectable in virtually all thymocytes of Gfi1<sup>GFP</sup>/GFP<sup>+</sup> and at significantly higher levels in thymocytes from Gfi1<sup>GFP/GFP</sup> mice (Fig. 1, c and d).

Next, we compared Gfi1 and GFP transcript levels in FACSSorted DN, DP CD4<sup>+</sup>, and CD8<sup>+</sup> thymocyte subsets from Gfi1<sup>+/OTPCR</sup> mice by real-time quantitative RT-PCR. Both the Gfi1 allele and the GFP knock-in allele showed similar but not identical mRNA expression levels (expressed as Δct values) in all four subsets indicating that the transcriptional regulation of the GFP knock-in allele remained largely intact after the homologous recombination at the Gfi1 gene locus (Fig. 1e). Analysis of Gfi1 protein levels in FACSSorted thymocyte subsets using a previously described α-Gfi1 antibody (9) revealed that two Gfi1 protein isoforms of 55 and 47 kDa exist that are both absent in Gfi1<sup>GFP/GFP</sup> knock-out cells (Fig. 1f). Both Gfi1 isoforms are expressed at highest levels in DN thymocytes and decrease in the DP and SP subsets (Fig. 1f). The 47-kDa Gfi1 isoform is expressed at slightly higher levels in CD4 and CD8 single positive thymocytes compared with the DP subset, whereas the 55-kDa isoform is lower in the SP populations than in DP or DN cells (Fig. 1f). GFP protein levels are also highest in DN cells and decrease in the DP subset and in CD4 and CD8 SP cells and, thus, follow the overall expression pattern of both Gfi1 isoforms (Fig. 1f) indicating that green fluorescence in Gfi1<sup>GFP</sup> cells can be a direct measure for Gfi1 expression.

Gfi1 Is Up-regulated during CD4/CD8 Lineage Decision and Pre-TCR Selection—To more precisely quantify the expression levels of Gfi1 during all steps of early T-cell development, we used MFI (mean fluorescence intensity) as a readout. Based on the expression of lineage markers (Lin) and the surface markers CD4, CD8, CD25, and CD44, thymocyte subsets of Gfi1<sup>+/GFP</sup> mice were electronically gated and analyzed for Gfi1 gene expression by measuring green fluorescence. Representative histogram analyses confirmed that Gfi1 is expressed in all stages of T-cell development in the DP and SP subsets as well as in the lineage negative (Lin<sup>−</sup>) DN subsets (Fig. 2a and b). The analyses showed further that Gfi1 gene expression increases during DN pre-T-cell development, reaches peak levels in the in β-selected “L” cells of the DN3 subset, and decreases again in DN4 and DP cells (Fig. 2, b and e, right panel). This pattern coincides with our previous assessment of Gfi1 protein levels in purified DN3, “E,” “L,” and DN4 cells (9). Interestingly, compared with total DP cells, Gfi1 gene expression was found to be up-regulated again in the CD4<sup>+</sup>CD8<sup>+</sup> population that undergoes positive/negative selection and where lineage decisions are made (Fig. 2c). Following the differentiation into the CD8 and CD4 direction, we noted that Gfi1 expression is maintained in CD8<sup>+</sup>CD4<sup>+</sup> but is down-regulated in CD4<sup>+</sup>CD8<sup>+</sup> cells (Fig. 2c, left and middle panel). In both CD4 and CD8 SP cells, Gfi1 expression decreases again compared with the CD4<sup>+</sup>CD8<sup>+</sup> population, but CD8<sup>+</sup> SP cells maintain a slightly higher Gfi1 expression level than CD4<sup>+</sup> SP cells (Fig. 2c, left and middle panel).

Gfi1 Expression Is Down-regulated during B-cell Maturation in Bone Marrow—The development of B-cells is well characterized and can be divided into specific stages based on the expression of B220 or CD19 surface markers in combination with c-Kit and CD43 (Fraction A and B/C, pre-pro-B cells, and pro-B cells) or CD25 (Fractions C and D, large and small pre-B-cells) or IgM/IgD (immature and mature B-cells) (Fig. 3a) (18, 19). The analysis of GFP fluorescence in different B-cell populations of Gfi1<sup>GFP</sup>/GFP<sup>+</sup> mice showed that the Gfi1 gene is expressed at earlier stages but that the expression largely decreases during further maturation of B-cells (Fig. 3, b and e, left panel) until GFP fluorescence and thus Gfi1 expression becomes undetectable in small, resting mature B-cells of the spleen (Fig. 3e). A more detailed inspection of the early B-cell compartment using expression of the markers CD43 and B220 showed that GFP fluorescence intensity is highest in Fraction A (pre-pro-B-cells), decreases in Fractions B/C (pro-B-cells), but rises again in Fraction C (large pre-B-cells) where the pre-BCR is assembled and presented on the cell surface (Fig. 3, c and e, right panel). Moreover, we separated the pre-B-cell subset into small resting (SR) and large cycling (LC) cells according to the expression of CD25 and B220 (2, 23) and found that, although both subsets express Gfi1, the expression level is significantly higher in LC than in SR cells (Fig. 3d).

Gfi1 Expression Is Up-regulated in Activated T-lymphocytes and Is Maintained at Different Levels in CD4 or CD8 Memory T-cells—In the spleen, GFP fluorescence was absent from small resting, mature peripheral B-lymphocytes (B220<sup>+</sup>) that were prepared by magnetic cell separation, but a low level of expression was detected in small resting T-lymphocytes (B220<sup>+</sup>CD3<sup>+</sup>) (Fig. 4a). Stimulation of B- and T-lymphocytes with LPS or α-CD3 antibodies for 24 h elicited a shift of green fluorescence to higher MFI values in both cell types indicating that Gfi1 gene expression can be significantly induced in peripheral B- and T-lymphocytes (Fig. 4a). Induction of GFP expression was about 7-fold in both B- and T-cell with respect to GFP levels in unstimulated cells (Fig. 4a).

Similar to our finding in thymocyte subsets, the quantification of Gfi1 and GFP transcript levels in T-cells during concanavalin A stimulation by real-time RT-PCR also demonstrated that the transcriptional regulation of the Gfi1 wt allele and the knock-in GFP allele is almost identical and was not
FIG. 1. Generation of Gfi1:GFP knock-in mice. a, after homologous recombination in ES cells and subsequent germ line transmission, the neomycin (Neo) gene was removed by mating to Cre-transgenic mice. \textit{loxP}, \textit{loxP} site; \textit{BamHI}; \textit{HindIII}; \textit{S}, Sacl; \textit{X}, XbaI; and \textit{E}, EcoRI. b, Southern blot analysis with the external 5'-probe depicted on the genomic locus of \textit{Gfi1} and BamHI- or HindIII-digested genomic DNA isolated from the indicated mice. After digestion with BamHI, the external 5'-probe (1 kb) detects a 7.5-kb fragment from the wt allele, a 14.6-kb fragment from the Gfi1\textsuperscript{GFP} allele, and a 13.2-kb fragment from the Gfi1\textsuperscript{GFP} allele. In a HindIII digest, this probe detects a 15-kb fragment from the wt allele, a 12-kb fragment from the Gfi1\textsuperscript{GFP} allele, and 10.6-kb fragment from the Gfi1\textsuperscript{GFP} allele. c, immunofluorescence analysis of total thymocytes from wt, Gfi1\textsuperscript{GFP} and Gfi1\textsuperscript{GFP} mice were fixed and stained with \textit{/H9251}-GFP antibodies and 4,6-diamidino-2-phenylindole. To detect GFP, cells were stained with a fluorescein isothiocyanate-labeled secondary antibody. All pictures were analyzed by laser scanning microscopy. d, GFP fluorescence in total thymocytes of wt, Gfi1\textsuperscript{GFP}, and Gfi1\textsuperscript{GFP} mice was detected by flow cytometry. The mean fluorescence intensity (MFI) from homozygous thymocytes (MFI = 47, \textit{n} = 11) was 3-fold higher than the MFI of heterozygous thymocytes (MFI = 14, \textit{n} = 8). e, quantification of mRNA levels transcribed from the \textit{Gfi1} allele (\textit{square}) and the GFP allele (\textit{open circle}) by real-time PCR. Shown are the \textit{ct} values, i.e. the number of cycles when the transcripts of \textit{Gfi1} and GFP genes becomes detectable on the same threshold line relative to glyceraldehyde-3-phosphate dehydrogenase. Four thymocyte subsets of Gfi1\textsuperscript{GFP} mice (\textit{DN}: CD4/CD8 double negative; \textit{DP}: CD4/CD8 double positive and CD4 or CD8 single positive cells) were analyzed. Given are mean values with standard deviations from analyses obtained from three independent Gfi1\textsuperscript{GFP} mice. f, \textit{Gfi1} and GFP protein levels in the indicated thymocyte subsets sorted from Gfi1\textsuperscript{GFP} mice or in total thymocytes from Gfi1\textsuperscript{GFP}, and Gfi1\textsuperscript{GFP} mice.
largely affected by the homologous recombination at the Gfi1 gene locus (Fig. 4b). When electronically separated CD4 and CD8 cells were stimulated, MFI values were induced 6- to 7-fold in CD4 cells but only about 3-fold in CD8 cells over unstimulated levels suggesting that induction of Gfi1 expression by TCR stimulation is more efficient in CD4 T-cells than in CD8 T-cells (Fig. 4c).

Gfi1 Autoregulation during T-cell Development—Our initial observation that thymocytes of Gfi1<sup>GFP/GFP</sup> mice show a severalfold higher MFI than the same cell population of Gfi1<sup>/+GFP</sup> mice (Fig. 1d) pointed to the possibility of an autoregulatory feedback loop at the Gfi1 locus. To examine this further, we introduced a Gfi1 transgene under the control of the proximal lck promoter into the Gfi1<sup>+/GFP</sup> mice by crossbreeding. lck-Gfi1 transgenic animals that were used for this purpose

---

**Fig. 2. Expression of Gfi1 during early T-cell development.** a, thymocytes from wt and Gfi1<sup>+/GFP</sup> mice were stained for expression of CD4 and CD8 and were gated as previously described (15, 17). Representative histogram showing the GFP fluorescence of the indicated subpopulations according to CD4/CD8 expression of wt mice (gray, filled) or Gfi1<sup>+/GFP</sup> animals (black line). b, analysis of double-negative thymocytes for GFP expression. A standard CD44-APC versus CD25-PE FACS staining is shown on previously electronically sorted Lin<sup>-</sup> thymocytes from wt mice to indicate the gates used for the definition of DN subsets. Representative histogram showing the GFP fluorescence of the indicated subpopulations according to CD25/CD44 expression of wt mice (gray, filled) or Gfi1<sup>+/GFP</sup> animals (black line). The DN3 subset was further separated into “E” and “L” cells as described previously (13, 15, 17). c, graphical presentation of GFP expression during T-cell development. Given are the MFIs of the indicated gated populations from Gfi1<sup>+/GFP</sup> animals as average values with standard deviations obtained from several analyzed animals (n = 8) and the same number of wt animals (Gfi1<sup>+/-</sup>).
had been described earlier and express constitutive high levels of Gfi1 in thymic and peripheral T-cells (5, 9). We observed clear shifts of green fluorescence to lower values in cells from lck-Gfi1/Gfi1GFP/GFP mice compared with cells from Gfi1GFP/GFP animals (Fig. 5a) suggesting a down-regulation of Gfi1 gene expression in the presence of the lck-Gfi1 transgene. The expression level of the GFP protein mirrored the flow cytometry data, because it increased in cells from Gfi1GFP/GFP mice and was almost entirely suppressed in Gfi1GFP/GFP carrying the lck-Gfi1 transgene (Fig. 5b). Gfi1 protein in Gfi1+/GFP was at wt levels but was absent from Gfi1GFP/GFP cells, as expected, because both Gfi1 alleles were knocked out (Fig. 5b). A nonspecific effect of the lck-Gfi1 transgene per se on the GFP expression could be excluded, because GFP expression in pre B-cells where the lck proximal promoter is inactive remained unaffected (data not shown) in both Gfi1+/GFP and Gfi1GFP/GFP mice.
To test whether this autoregulation is restricted to a specific T-cell type, we analyzed to what extent green fluorescence was shifted to lower levels in thymic and peripheral T-cell subsets of Gfi1<sup>GFP/GFP</sup> mice in the presence of the lck-Gfi1 transgene. Although a nearly complete silencing of the Gfi1 gene locus was observed in single positive CD4 or CD8 cells from both thymus...
and spleen (Fig. 5, c and e), the degree of Gfi1 autoregulation and silencing of the Gfi1 allele was only partial in the DP cell subset or incomplete as in the CD4loCD8lo, CD8l/H11001CD4lo, and CD4l/H11001CD8lo subsets (Fig. 5c). In these latter subpopulations, two groups of cells could be distinguished: one group showed almost full silencing of the Gfi1 locus, whereas in the other group, the Gfi1 locus was only partially silenced or remained fully transcriptionally active despite the presence of the lck-Gfi1 transgene (Fig. 5c). Similarly, the different DN subsets did not all show the same autoregulatory behavior. In DN2 cells, the Gfi1 locus was not silenced at all; DN1 and DN3 cells showed a partial silencing and DN4 cells contain a minor fraction that shows a down-regulation of Gfi1 transcription, while the majority maintained Gfi1 gene expression levels despite the presence of the lck-Gfi1 transgene (Fig. 5d).

The Gfi1 promoter has been previously defined by the genomic region immediately upstream of the 5’ end of the Gfi1 cDNA (2). Our own rapid amplification of cDNA ends and primer extension experiments confirmed the location of the Gfi1 promoter (Fig. 6a, and data not shown). Inspection of the genomic region of the Gfi1 promoter using the program MathInspector (24) revealed the existence of several “AATC” Gfi1 binding consensus sites in the 5’ to 3’ direction (Fig. 6a). To investigate whether the autoregulation of Gfi1 is mediated by a direct mechanism of transcriptional repression, we isolated the proximal part of the Gfi1 promoter sequence by PCR (Fig. 6a) and appended it to a luciferase reporter gene. In NIH 3T3 cells, the activity of the Gfi1 reporter construct could be significantly repressed in a concentration-dependent manner by co-expression of Gfi1 (Fig. 6b, upper panel) supporting an
FIG. 6. Analysis of the Gfi1 promoter reveals an autoregulatory feedback loop of Gfi1 transcription. a, DNA sequence from the genomic region immediately upstream of the Gfi1 coding region (boxed). The transcription start site was determined by rapid amplification of cDNA ends or primer extension experiments (data not shown) and are indicated by arrowheads and asterisks, respectively. The start sites coincide with or are very close to the 5' end of the published Gfi1 cDNA (2). Putative Gfi1 binding sites in the 5' → 3' direction (AATC) and in 3' → 5' direction (GATT) are indicated in bold letters and are underlined. b, a DNA fragment spanning the region of the Gfi1 promoter from position 1 to position 593 was amplified by PCR using the primers Gfi1-P and Gfi1-P as indicated in a. This fragment was inserted into the pGL3 basic vector to generate a Gfi1 promoter-driven luciferase reporter gene construct. Transfection into NIH 3T3 cells revealed promoter activity that was repressed in the presence of a functional Gfi1 protein in a concentration-dependent manner (upper panel). Repression was not observed when the non-functional Gfi1-P2A mutant (P2A) described previously (6) or a mutant lacking the zinc finger domains (ΔZn, lower panel) was used. c, thymocytes from wt (Gfi1+/+) or Gfi1−/− mice (17) were isolated and treated with glutaraldehyde to cross-link DNA and DNA-binding proteins. After sonication, DNA–protein complexes were precipitated with an α-Gfi1 antibody that has been described previously (29), and the precipitated DNA fragments were amplified with the indicated primer pairs (Gfi1-I, Gfi1-II, and Gfi1-III, or the irrelevant primer pair CTLA4). The amplification reactions were separated on agarose gels and visualized by a negative image of ethidium bromide staining.
autoregulatory mechanism at the Gfi1 locus. Such a repression was not observed with mutants of Gfi1 that either lack the zinc finger domains (∆Zn) or carry a single amino acid exchange mutation in the SNAG domain (P2A) (Fig. 6b, lower panel) suggesting that the autorepression of Gfi1 depends on both the DNA binding ability of Gfi1 and an intact SNAG domain. In addition, to test whether Gfi1 indeed binds to sequences within its own promoter in vivo, chromatin immunoprecipitation experiments using freshly isolated thymocytes from wt mice (Gfi1+/−) and Gfi1-deficient animals (Gfi1−/−) as a control were performed. Immunoprecipitated DNA fragments were amplified by PCR using Gfi1-specific primer pairs Gfi-I, Gfi-II, and Gfi1-III, which contain sequences of the Gfi1 promoter region, but not with a primer pair that contains sequences from the unrelated CTLA4 promoter (Fig. 6c). With all three Gfi1 promoter-specific primer sets and with approximately similar input levels, Gfi1-DNA complexes could be clearly detected from Gfi1+/− thymocytes in comparison to background levels obtained with Gfi1−/− cells (Fig. 6c). No signal was obtained with the irrelevant primer pair from the CTLA4 promoter region demonstrating that Gfi1 indeed directly occupies its cognate binding sites that are located in its own promoter region (Fig. 6c).

**DISCUSSION**

We have used a GFP knock-in strategy to follow the expression of the transcription factor Gfi1 in vivo in particular during the development of T- and B-lymphocytes. To create a system that would faithfully mirror the transcriptional activity of the Gfi1 locus, we have inserted the coding region of a GFP reporter gene into the Gfi1 locus precisely at the translation initiation codon of Gfi1. Thus, the GFP reporter gene was placed directly under the transcriptional control of the upstream regulatory sequences of the Gfi1 gene. In addition, the 3′ termination signals, including the 3′ untranslated region and the polyadenylation site of the Gfi1 gene locus, were also left intact to ensure that the transcription of the Gfi1:GFP knock-in allele most closely resembles the Gfi1 wt allele. Indeed, quantitative real-time RT-PCR analysis of thymocyte subsets and peripheral T-cells before and after activation demonstrated clearly that the transcriptional regulation of the GFP knock-in allele and very likely also the half-life of the GFP-specific transcript remained largely intact after homologous recombination and is very similar but not identical to the wt Gfi1 allele. In addition, Gfi1 and GFP protein levels follow a similar regulation during early T-cell development indicating that Gfi1:GFP knock-in mice represent a valid model to follow Gfi1 expression in different cell population or organs of a mouse.

In addition, GFP expression very closely followed the already known expression pattern of Gfi1 in peripheral T-cells, i.e. GFP fluorescence was found to be higher in pre-T-cells of the thymus than in peripheral T-cells and was strongly up-regulated upon antigenic TCR stimulation. These features had already been determined by other methods (8, 9), and the congruency of our findings with these earlier results suggests that our model correctly mimics the expression pattern of Gfi1 and can be used to follow Gfi1 expression in vivo by measuring green fluorescence. Interestingly, we observed that in Gfi1+/−/GFP thymocyte subsets both Gfi1- and GFP-specific transcripts levels, which are regulated very similarly, do not coincide with the respective Gfi1 or GFP protein levels. For instance, in thymocytes of Gfi1+/−/GFP mice, Gfi1 or GFP mRNA expression is higher in the DP subset than in DN cells, but Gfi1 or GFP protein expression levels in the DP subpopulation are clearly lower than in the DN subset. It is therefore likely that, at least in thymocytes of Gfi1+/−/GFP mice, green fluorescence mirrors the level of Gfi1 protein more faithfully than the level of Gfi1-specific mRNA. However, our model also has clear limitations, which became apparent when two Gfi1 isoforms were detected in the Western analysis of FACS-sorted thymocyte subsets. Although the expression level of both the 55- and 47-kDa forms was highest in DN cells and declined in DP and further in CD4 and CD8 SP cells, the smaller isoform showed slightly different kinetics than the larger isoform. This complexity cannot be accurately represented by GFP fluorescence in our Gfi1:GFP knock-in mouse and has to be further analyzed by other methods. In addition, the knock-in of the GFP coding region into the Gfi1 gene locus was performed by deleting considerable stretches of Gfi1 sequences, including introns. Because it has been shown that a Gfi1 binding site, which is located in the first intron, contributes to the regulation of the Gfi1 locus (30), it cannot be excluded that our targeting has also deleted other regulatory sites in other parts of the Gfi1 gene, possibly in introns, which might explain the slight divergence of Gfi1 and GFP mRNA expression levels.

In all tested subpopulations from the thymic DP and the DN compartment, virtually no heterogeneity was observed and all cells appeared to express GFP. DN1 and the DN2 subset may be the only exception to this rule, because in these subsets a very small subpopulation seemed to be GFP-negative. DN1 and DN2 cells are uncommitted, require cytokines such as stem cell factor (c-Kit-ligand) and interleukin-7 for growth and survival (25, 26), carry unarranged germ line TCR alleles, and still bear the potential to develop into other lineages such as thymic dendritic cells, NK cells, or B-cells. Lack of Gfi1 expression among these subsets may thus correlate with a non-T-cell differentiation commitment. Another finding that is of interest with regard to pre-T-cell development is the continuous increase in expression of Gfi1 during DN differentiation until the pre-TCR selection takes place in the DN3 “L” cell subset where Gfi1 expression reaches peak levels. These cells have productively rearranged their TCRβ gene and are strongly proliferating, thereby producing cells for the subsequent differentiation steps (12, 13). Compared with other early and late T-cell subpopulations, Gfi1 expression appears to be higher during pre-TCR selection and in β-selected cells than in all other T-cells even in those stimulated with antigen. This high transcriptional activity of the Gfi1 locus in β-selected cells supports earlier speculations about an essential role of Gfi1 in this process. Gfi1-deficient mice have significantly lower numbers of DN3 cells and show features consistent with a faster passage through this differentiation stage (17). In contrast, transgenic mice that overexpress Gfi1 by virtue of the T-cell-specific proximal lck promoter have higher DN3 percentages than wt mice and have strongly reduced numbers of L-cells (9). In neither case, however, is an entire block of pre-TCR selection observed, thus suggesting that Gfi1 takes part in this process but is not an indispensable factor.

Interestingly, Gfi1 expression also rises to higher levels in pre B-cells, which carry a pre-BCR and are considered to be the equivalent to DN3 cells expressing a pre-TCR selection (18, 19). This suggests a similar or identical mechanism of transcriptional regulation of the Gfi1 gene by pre-T and pre-B cell receptor-mediated signals and points to a similar role of Gfi1 in both the early T- and early B-cell selection steps. By receptor cross-linking, Gfi1 expression can be quickly induced, which is witnessed by an up-regulation of green fluorescence in both peripheral B- and T-cells from GFP knock-in mice. This points to the possibility that the transcriptional regulation of the Gfi1 allele is the end point of the same signaling events in both types of lymphocytes triggered by either the T-cell or the B-cell receptor.
A transient increase of Gfi1 expression confined to a cellular subset that is under selection is also observed in the more mature DP compartment of the thymus. Here, the CD4CD8^lo cells that are still largely uncommitted (8), but most probably represent the majority of cells that are under positive/negative selection, show peak levels of Gfi1 expression within the DP subset. Considering that Gfi1 expression is highest in cells where pre-TCR signaling occurs and is stimulated in peripheral T-cells upon antigenic stimulation, it is conceivable that Gfi1 expression in general follows stimuli from TCR-mediated signals and that in the CD4^loCD8^lo population MHC-TCR-TCR interaction provides the trigger that up-regulates Gfi1 expression. Interestingly, the cells that develop from the CD4^loCD8^lo population to become CD4 SP cells by down-regulation of CD8 also significantly down-regulate Gfi1 expression. By contrast, cells that have to re-express CD8 and shut down CD4 to develop into CD8 SP cells maintain Gfi1 expression in an intermediate CD8^-CD4^lo population before Gfi1 expression decreases in CD8 SP cells. This suggests that transcription of the Gfi1 gene is differentially regulated during the process of lineage decision or positive selection depending on the type of co-receptor molecule present. One interpretation of this finding could be that Gfi1 expression levels mirror the signal quality of TCR-MHC engagement. It has been proposed that signals from TCR interactions with class I or class II MHC are of different intensity and that the "weaker" TCR-MHC class I signal directs cells into the CD8 lineage while the stronger TCR-MHC class II signal triggers the formation (or selection) of CD4 SP cells (27). In an alternative model, CD4/CD8 lineage determination and positive selection depend, rather, on the signal persistence and not the strength. According to this hypothesis, CD4 SP cells are selected from the DP pool by persistent TCR-MHC-mediated signals and CD8 SP cells develop from a CD4^-CD8^- cell intermediate subset upon cessation of the TCR signal by co-receptor reversal and re-expression of CD8 (28, 29). Although it remains open which model more accurately reflects the physiological situation, our present results together with previously described findings suggest that Gfi1 is one of the downstream effectors of TCR-MHC signaling that may help to regulate the CD4/CD8 lineage decision and positive/negative selection.

A difference between CD4 and CD8 SP in Gfi1 expression levels as seen in the thymus is no longer maintained in quiescent, peripheral CD4^- or CD8^- T-cells in spleen or lymph nodes. Here, the expression levels according to MFI values are lowest compared with all other T-cell subsets and only rise upon treatment with α-CD3 antibodies but to different levels in CD4 or CD8 naive or memory subsets. The molecular mechanism of this differential regulation remains to be determined but particularly the divergence of Gfi1 transcriptional activity between CD4 or CD8 memory cell subtypes points to a potentially novel function of Gfi1 in the establishment of immunological memory that deserves future attention.

The initial analysis of Gfi1^GFP/GFP thymocytes and the comparison of GFP levels with those measured in Gfi1^-GFP cells suggested an autoregulatory feedback loop for Gfi1. Chromatin immunoprecipitation and reporter gene assays provided strong evidence that Gfi1 can physically bind to its own promoter sequence, that these sites are indeed occupied by Gfi1 in vivo, and that this ensues transcriptional repression of the Gfi1 allele. Moreover, our analyses with combinatorial lck-Gfi1/Gfi1^GFP/GFP mice further support the existence of Gfi1 autoregulation in T-cells, because MFI values are dramatically reduced until an almost complete silencing of the Gfi1 gene locus in the presence of an Gfi1 transgenic allele. Autoregulation of Gfi1 by a direct transcriptional feedback mechanism seems therefore likely. Additional support for this also comes from very recent findings using T-cell lines and transgenic mice constitutively expressing Gfi1 in T-cells similar to the ones described here (30).

Interestingly, in different subsets of T-cells the silencing of the Gfi1 allele by the lck-Gfi1 transgene is variable being complete in one subpopulation and partial or incomplete in others. This could be the result of a cell type-specific autoregulation of the Gfi1 allele, or more likely, could reflect higher or lower expression levels of the lck-Gfi1 transgene in the different T-cell subpopulations. The latter would be consistent with our reporter gene assays, which demonstrate that the silencing of the Gfi1 promoter by Gfi1 is dose-dependent, and with recent findings with an lck-GFP transgenic mouse line. In these animals, the activity of the proximal lck promoter was found to be most active in thymic CD4 and CD8-SP cells, less active in DP cells, and even weaker in DN cells (31), which precisely matches our observations of GFP fluorescence reduction in the respective thymic subsets of lck-Gfi1/Gfi1^GFP/GFP mice.

In summary, we have created a mouse model that in future experiments will allow the close monitoring in vivo of the expression of Gfi1, which has previously been shown to play important roles in the acquired and innate immune response. Possible applications would be the monitoring of Gfi1 gene expression as a response to particular signaling pathways, to immunization, or during the differentiation pathways that lead to the formation of Th1 and Th2 cells. Moreover, it will be of interest to see how the Gfi1 gene responds to B-cell activation and during differentiation to plasma cells in vivo. Lastly, because loss of Gfi1 ensues a severe neutropenia and an exaggerated inflammatory reaction (20, 22) toward pathogen-associated molecular patterns, our model will be most useful to determine the transcriptional activity on the Gfi1 allele during granulo/monocyctic differentiation, macrophage activation, and inflammatory processes in general.

Acknowledgments—We are indebted to Angelika Warda, Wojciech Wegryn, Inge Spratte, Klaus Lennartz, and Adriane Parchatka for technical assistance and Petra Plessow and Tomas Civela for excellent animal care.

REFERENCES

1. Gilks, C. B., Bear, S. E., Grimes, H. L., and Tsichlis, P. N. (1993) Mol. Cell. Biol. 13, 1759–1768
2. Scheijen, B., Jonkers, J., Acton, D., and Berns, A. (1997) J. Virol. 71, 9–16
3. Schmidt, T., Zornig, M., Beneke, R., and Muroy, T. (1996) Nucleic Acids Res. 24, 2528–2534
4. Zornig, M., Schmidt, T., Karsunky, H., Grzeschik, A., and Moroy, T. (1996) Oncogene 12, 1789–1905
5. Schmidt, T., Karsunky, H., Gau, E., Zevnik, B., Elssasser, H. P., and Moroy, T. (1998) Oncogene 17, 2661–2667
6. Grimes, H. L., Chai, T. O., Zweidler-Mckay, P. A., Tong, B., and Tsichlis, P. N. (1996) Mol. Cell. Biol. 16, 6263–6273
7. Zweidler-Mckay, P. A., Grimes, H. L., Flubacher, M. M., and Tsichlis, P. N. (1996) Mol. Cell. Biol. 16, 4024–4034
8. Karsunky, H., Mende, I., Schmidt, T., and Moroy, T. (2002) Oncogene 21, 1571–1579
9. Schmidt, T., Karsunky, H., Rodel, B., Zevnik, B., Elssasser, H. P., and Moroy, T. (1998) EMBO J. 17, 5349–5359
10. Feihling, H. J., and von Boehmer, H. H. (1997) Curr. Opin. Immunol. 9, 263–275
11. Godfrey, D. I., Kennedy, J., Suda, T., and Zlotnik, A. (1993) J. Immunol. 150, 4444–4452
12. Dudley, E. C., Petri, H. T., Shah, L. M., Owen, M. J., and Hayday, A. C. (1994) Immunol. 1, 83–93
13. Hoffmann, E. S., Passoni, L., Crompton, T., Leu, T. M. U., Schutz, D. G., Koff, A., Owen, M. J., and Hayday, A. C. (1996) Nat. Genet. 5, 309–322
14. Saio, T., and Watanabe, N. (1998) Crit. Rev. Immunol. 18, 359–370
15. Yu ¨ cel, R., Karsunky, H., Grzeschik, A., Moroy, T., and Muroy, T. (2003) J. Exp. Med. 197, 831–844
16. Hardy, R. R., and Hayakawa, K. (2001) Ann. Rev. Immunol. 19, 595–621
17. Ceredig, R., and Rolink, T. (2002) Nat. Rev. Immunol. 2, 2–10
18. Karsunky, H., Zornig, M., Schmidt, T., Zevnik, B., Kluge, R., Schmid, K. W., Dohren, U., and Moroy, T. (2002) Nat. Genet. 30, 295–300
19. Orlando, V., Strutt, H., and Paro, R. (1997) Methods 11, 205–214
20. Hock, H., Hamblen, M. J., Rothe, H. M., Traver, D., Bronson, R. T., Cameron,
Gfi1:GFP Knock-in Mice

S., and Orkin, S. (2003) Immunity 18, 109–120
23. Monroe, R. J., Seidl, K. J., Gaertner, F., Han, S., Chen, F., Sekiguchi, J. A., Wang, J., Ferrini, R., Davidson, L., Kelseo, G., and Alt, F. W. (1999) Immunity 77, 201–212
24. Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995) Nucleic Acids Res. 23, 4878–4884
25. Murray, R., Suda, T., Wrighten, N., Lee, F., and Zlotnik, A. (1998) Int. Immunol. 1, 526–531
26. Baird, A. M., Gerstein, R. M., and Berg, L. J. (1999) Curr. Opin. Immunol. 11, 157–166
27. Hogquist, K. A. (2001) Curr. Opin. Immunol. 13, 225–231
28. Bosselut, R., Feigenbaum, L., Sharrow, S. O., and Singer, A. (2001) Immunity 4, 483–494
29. Barthlett, T., Kohler, H., and Eichmann, K. (1997) J. Exp. Med. 185, 357–362
30. Dean, L. L., Porter, S. D., Duan, Z., Fluhacker, M. M., Montoya, D., Tsichlis, P. N., Horwitz, M., Gilks, C. B., and Grimes, H. L. (2004) Nucleic Acids Res. 32, 2508–2519
31. Shimizu, C., Kawamoto, H., Yamashita, M., Kimura, M., Konodou, E., Kaneko, Y., Okada, S., Tokuhisa, T., Yokoyama, M., Taniguchi, M., Katsura, Y., and Nakayama, T. (2001) Int. Immunol. 13, 105–117
Gfi1:Green Fluorescent Protein Knock-in Mutant Reveals Differential Expression and Autoregulation of the Growth Factor Independence 1 (Gfi1) Gene during Lymphocyte Development

Raif Yücel, Christian Kosan, Florian Heyd and Tarik Möröy

J. Biol. Chem. 2004, 279:40906-40917.
doi: 10.1074/jbc.M400808200 originally published online July 13, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M400808200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 9 of which can be accessed free at http://www.jbc.org/content/279/39/40906.full.html#ref-list-1