MOLECULAR AND CELLULAR BIOLOGY, Nov. 1996, p. 6263–6272 Vol. 16, No. 11
0270-7306/96/$04.00+0

The Gfi-1 Proto-Oncoprotein Contains a Novel Transcriptional Repressor Domain, SNAG, and Inhibits G_1 Arrest Induced by Interleukin-2 Withdrawal

H. LEIGHTON GRIMES, TUNG O. CHAN, PATRICK A. ZWEIDLER-MCKAY, BETTY TONG, AND PHILIP N. TSICHLIS*

Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Received 17 June 1996/Returned for modification 22 July 1996/Accepted 7 August 1996

The *Gfi*-1 proto-oncogene is activated by provirus insertion in T-cell lymphoma lines selected for interleukin-2 (IL-2) independence in culture and in primary retrovirus-induced thymomas and encodes a nuclear, sequence-specific DNA-binding protein. Here we show that Gfi-1 is a position- and orientation-independent active transcriptional repressor, whose activity depends on a 20-amino-acid N-terminal repressor domain, coincident with a nuclear localization motif. The sequence of the Gfi-1 repressor domain is related to the sequence of the repressor domain of Gfi-1B, a Gfi-1-related protein, and to sequences at the N termini of the insulinoma-associated protein, IA-1, the homeobox protein Gsh-1, and the vertebrate but not the *Drosophila* members of the Snail-Slug protein family (Snail/Gfi-1, SNAG domain). Although not functionally characterized, these SNAG-related sequences are also likely to mediate transcriptional repression. Therefore, the Gfi-1 SNAG domain may be the prototype of a novel family of evolutionarily conserved repressor domains that operate in multiple cell lineages. Gfi-1 overexpression in IL-2-dependent T-cell lines allows the cells to escape from the G_1 arrest induced by IL-2 withdrawal. Since a single point mutation in the SNAG domain (P2A) inhibits both the Gfi-1-mediated transcriptional repression and the G_1 arrest induced by IL-2 starvation, we conclude that the latter depends on the repressor activity of the SNAG domain. Induction of Gfi-1 may therefore contribute to T-cell activation and tumor progression by repressing the expression of genes that inhibit cellular proliferation.

The signals initiating the process of T-cell activation are transmitted from the cell membrane to the nucleus through a series of phosphorylation events which lead to the expression of interleukin-2 (IL-2) and its high-affinity receptor. Interaction of IL-2 with its receptor is a critical step coinciding with the commitment to later T-cell activation events (11). To study the regulation of T-cell activation, we have chosen to use an insertional mutagenesis-based genetic strategy which was designed to identify genes involved in the progression of IL-2-dependent T-cell lymphoma lines to IL-2 independence. The *Gfi*-1 gene, which was cloned by this strategy, encodes a zinc finger protein with six C_2H_2-type, tandem zinc finger motifs located in the C-terminal half of the protein. Expression of Gfi-1 in IL-2-dependent cells following transfer of a *Gfi*-1/LXSN retrovirus construct facilitated the isolation of IL-2-independent cell lines (13).

Gfi-1 expression in adult animals is restricted to the thymus, spleen, and testis. In mitogen-stimulated splenocytes, *Gfi*-1 expression begins to rise at 12 h after stimulation and reaches very high levels after 50 h, suggesting that Gfi-1 may be functionally involved in events occurring after the interaction of IL-2 with its receptor. In agreement with this, *Gfi*-1 does not induce the expression of IL-2 (13).

Gfi-1 contributes not only to the selection of IL-2-dependent T-cell lymphoma lines for IL-2 independence but also to the induction and progression of several types of rodent hematopoietic neoplasms. *Gfi*-1 is a locus of common proviral integration in T-cell tumors induced by Moloney murine leukemia virus (MoMuLV) (13), mink-cell focus-forming virus (27), and murine acquired immunodeficiency virus (34). Transgenic mice in which either the c-*myc* or *pim*-1 oncogene is expressed from the *pim*-1 promoter with the immunoglobulin heavy-chain enhancer (Eμ) develop B- and T-cell lymphomas at an accelerated rate after MoMuLV inoculation. These lymphomas carry provirus insertions at a locus, *pal*-1, which is synonymous with *Gfi*-1 (6). Another locus targeted by MoMuLV in these mice is *Bmi*-1, which encodes a polycomb-like protein (6). However, insertions at the *Bmi*-1 locus and insertions in *Gfi*-1 are mutually exclusive. Therefore, *Gfi*-1 and *Pim*-1 or c-*myc* appear to exhibit functional complementarity, while *Gfi*-1 and *Bmi*-1 may be functionally redundant (6). In addition to the development of rodent hematopoietic tumors, *Gfi*-1 may contribute to the induction and progression of human neoplasms. The human homolog of *Gfi*-1 has been mapped to human chromosome 1p22, a region of nonrandom chromosomal abnormalities in patients with human non-Hodgkin's lymphomas, mesotheliomas, malignant melanomas, bladder and mammary neoplasms, germ cell (yolk sac) tumors, pheochromocytomas, and pleomorphic adenomas of the salivary gland (5).

We recently reported that Gfi-1 is a nuclear protein that binds a 12-bp DNA sequence containing an AATC or AAGC core (38). Mutations in the core abrogated Gfi-1 binding in vitro. Moreover, deletion of individual zinc fingers revealed that only fingers 3, 4, and 5 are required for sequence-specific DNA binding. Gfi-1 transfected transiently into NIH 3T3 cells induced the repression of a cotransfected chloramphenicol acetyltransferase (CAT) reporter construct driven by the major immediate-early (MIE) promoter of the human cytomegalovirus (CMV), suggesting that Gfi-1 encodes a transcriptional repressor (38).

The experiments discussed in this report were designed to

* Corresponding author. Mailing address: Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111. Phone: (215) 728-3635. Fax: (215) 728-2741.

address whether the endogenous Gfi-1, expressed naturally in T cells, also functions as a transcriptional repressor and to characterize the Gfi-1-mediated repression mechanism. Potential mechanisms responsible for repression include (i) competition between repressors and activators for binding the same DNA site; (ii) repressor-mediated blocking of the contact of an activator with the basic transcriptional complex, also called short-range repression or quenching; and (iii) repressor-mediated inhibition of the basic transcription machinery, also called active transcriptional repression (20).

The results showed that Gfi-1 encodes a position- and orientation-independent active transcriptional repressor that functions in both fibroblasts and T cells. Transcriptional repression by Gfi-1 is mediated by an amino-terminal, 20-amino-acid novel repressor domain, coincident with a nuclear localization motif. The Gfi-1 repressor domain is shared by Gfi-1, Gfi-1B (a Gfi-1-related protein), the orphan Hox gene Gsh-1, the insulinoma-associated protein IA-1, which is induced during the progression stage of human insulinomas and other neuroendocrine neoplasms including small cell lung carcinomas (23), and the vertebrate (but not the *Drosophila*) members of the Snail-Slug protein family (Snail/Gfi-1, SNAG domain). These data indicate that Gfi-1 identifies a novel family of transcriptional repressors.

Overexpression of Gfi-1 from a heterologous promoter releases cells from a G_1 arrest induced by growth factor withdrawal in the IL-2-dependent T-cell lymphoma line 2780a. A single amino acid substitution in the Gfi-1 SNAG domain not only impairs the repressor activity of the cognate protein but also fails to rescue G_1-arrested, IL-2 deprived 2780a cells. Therefore, the Gfi-1 mediated SNAG repression and the cell cycle effects of Gfi-1 appear to be linked.

MATERIALS AND METHODS

Antibodies (production and affinity purification), Western blotting, immunofluorescent confocal microscopy. Two multiple-antigen peptides, corresponding to Gfi-1 amino acid sequences 12 to 26 and 336 to 350, were synthesized (Research Genetics, Huntsville, Ala.). The multiple-antigen peptides were used to immunize rabbits at the Fox Chase Cancer Center animal facility, and reactive antisera were affinity purified by standard methods (18) with columns of Sepharose-bound multiple-antigen peptides. Western blotting (immunoblotting) was carried out with Immobilon P membranes (Amersham). Membrane-bound proteins were detected by enhanced chemiluminescence (Kodak). Immunofluorescent staining was carried out by standard procedures (18). Stained cell monolayers were processed at the Fox Chase Confocal Microscopy facility.

Plasmid construction. Reporter constructs were generated by modification of the pCAT Basic plasmid (Promega). Briefly, to generate the base vector C.2, we first mutated the plasmid backbone by USE mutagenesis (12) to eliminate potential Gfi-1-binding sites. We then introduced a *Hin*dIII restriction site 3′ of the CAT gene by replacing the *Eco*RI fragment with the equivalent fragment from the TKCAT plasmid, pBLCAT2 (29). Finally, 5′ of the CAT gene, we cloned an *Eco*RV, *Bgl*II, and *Bam*HI site containing double-stranded oligonucleotide. Oligonucleotides required for these manipulations were synthesized by the Fox Chase DNA synthesis facility. A pBLCAT2 *Bam*HI-*Bgl*II fragment containing the herpes simplex virus thymidine kinase (tk) promoter was then cloned into the *Bam*HI site, and Gfi-1-binding sites (r21 or b30) or a single LexA operator was inserted by cloning double-stranded oligonucleotides into the *Bgl*II-*Bam*HI-digested vector. To generate the simian virus 40 (SV40)-CAT construct with two b30 oligonucleotides, the tk-CAT cassette was excised with *Bam*HI-*Hin*dIII, and the corresponding *Bgl*II-*Hin*dIII SV40-CAT cassette from the pCAT promoter (Promega) was inserted. All products of cloning and PCR mutagenesis used in this study were sequenced to ensure that no additional mutations were generated. The β-galactosidase expression construct, cotransfected to normalize for transfection efficiency, contains a mutant CMV MIE promoter which is no longer responsive to Gfi-1 repression (mutant B [38]).

Gfi-1 expression constructs were generated by cloning the *Eco*RI-*Bam*HI fragment of the C2B cDNA into the CMV5 expression vector (2). Mutants of Gfi-1 were generated by PCR and overlap extension PCR. The delta *Eco*47III mutant was generated by digestion with *Eco*47III followed by religation. The deletion mutants lack the following amino acids: delta 1, amino acids 21 to 67; delta 2, amino acids 68 to 114; delta 3, amino acids 115 to 161; delta 4, amino acids 162 to 208; delta 5, amino acids 209 to 255; delta exon 3, amino acids 39 to 99; delta *Eco*47III, amino acids 149 to 194; and delta non-ZN, amino acids 22 to 251. The SV40ZN and SV40 swap contain the SV40 nuclear localization signal (NLS) fused to Gfi-1 amino acid 252 or 21, respectively. The SV40 nuclear localization signal and a translation initiation sequence (MGAPPKKKRKVA) were derived from pJG4-5 (16). The delta ZN mutant was constructed by cloning a double-stranded oligonucleotide encoding a hemagglutinin epitope tag, an *Xho*I restriction site, and a stop codon into the *Nsi*-1 and *Bam*HI sites of the C2B Gfi-1 cDNA.

To generate the LexA expression constructs, the *lexA*-containing *Eco*RI-*Sal*I fragment of pSN203 (a gift of Steve Nottwehr and Manuel Sainz) was cloned into the CMV5 vector. The Gfi-1/LexA fusion construct was generated by cloning the *Eco*RI-*Xho*I Gfi-1 delta ZN DNA fragment 5′ of and in frame with the *lexA* gene.

The SRα/Gfi-1 virus was constructed as follows. The TGA codon of the C2B cDNA of Gfi-1 (in pBluescript) was mutated to a *Bam*HI site via overlap extension PCR, after which double-stranded oligonucleotides, encoding the FLAG epitope (Kodak) and six histidines followed by a TGA termination codon and an *Eco*RI site, were inserted between the new *Bam*HI site and the one in the 3′ untranslated region. This manipulation generated an *Eco*RI-*Eco*RI fragment (encoding a tagged version of Gfi-1), which was cloned into the *Eco*RI site of the SRα virus vector (24).

Cell lines, transient transfections, and CAT assays. Jurkat clone E6.1 (TIB 152), EL4.IL2 (TIB 181), and NIH 3T3 (TIB 163) were obtained from the American Type Culture Collection (Rockville, Md.). Plasmid DNA was prepared on Qiagen columns by procedures suggested by the manufacturer. Electroporations of lymphoid cells were carried out with 20 μg of DNA, 0.45-μm cuvettes (Bio-Rad), and 10^6 cells suspended in 250 μl of media (without antibiotics). Samples were pulsed at 960 μF and 270 mV and placed in culture for 36 h before being harvested. NIH 3T3 cells were transfected with Lipofectamine reagent (BRL Gibco) exactly as suggested by the manufacturer. Cell lysates were generated by consecutive freeze-thaw cycles and were normalized for transfection efficiency by a microtiter plate-based β-galactosidase assay (7). Lysates were analyzed for CAT activity by either thin-layer chromatography or diffusion of the acetylated form in scintillation fluid as described previously (38). Both methods gave equivalent results. All transfections were performed at least in triplicate for NIH 3T3 cells and six times for EL4 and Jurkat cells and were repeated at least twice with different preparations of plasmid DNA.

SRα retrovirus constructs were packaged into retrovirus particles by Lipofectamine transfection into 293T cells as described previously (24).

RESULTS

Gfi-1 binding sites are sequence-specific, *cis*-acting, distance-independent repressor elements. We had previously shown that *Gfi*-1 mRNA is expressed in adult rat thymus, spleen, and testis as well as in most MoMuLV-induced rat T-cell lymphomas (13). Here we show that two additional T-cell lymphoma lines, Jurkat and EL4, also express *Gfi*-1 as determined by Northern (RNA) blot analysis (data not shown) and immunoprecipitation (Fig. 1A). To test whether endogenous Gfi-1 functions as a transcriptional regulator in lymphoid cells, the last two cell lines were electroporated with reporter constructs containing the bacterial CAT gene driven by the herpes simplex virus tk promoter. One to four copies of the Gfi-1-binding-site oligonucleotides r21 or b30 (Fig. 1B) were cloned 5′ of the tk promoter, and four to eight copies of the same oligonucleotides were cloned 3′ of the CAT gene (Fig. 1C). CAT assays with extracts of the transfected cells revealed that both binding sites function as *cis*-acting repressor elements and that the repression is additive (Fig. 1D, lanes 1 to 4 and 7 to 10). Moreover, the repression induced by four sites cloned 3′ of the tk-CAT cassette is equivalent to the repression induced by one site cloned 5′ (Fig. 1D, lanes 2, 6, 8, and 12). Therefore, the Gfi-1-binding sites can induce repression of the tk promoter from a distance. The Gfi-1-mediated repression is not limited to the tk promoter, because reporter constructs demonstrate repressed levels in EL4 and Jurkat cells when two b30 sites are cloned 5′ of the SV40 promoter (data not shown).

Five clones in the random-oligonucleotide-binding-site selection contained AAGC cores in addition to AATC (38). Mutation of the AATC core of the B30 site to AAGC had no effect on repression (data not shown), indicating that Gfi-1 may also function through binding sites with AAGC core sequences. In contrast, the repression of both the r21 tk-CAT and b30 tk-CAT reporter constructs was abolished when the

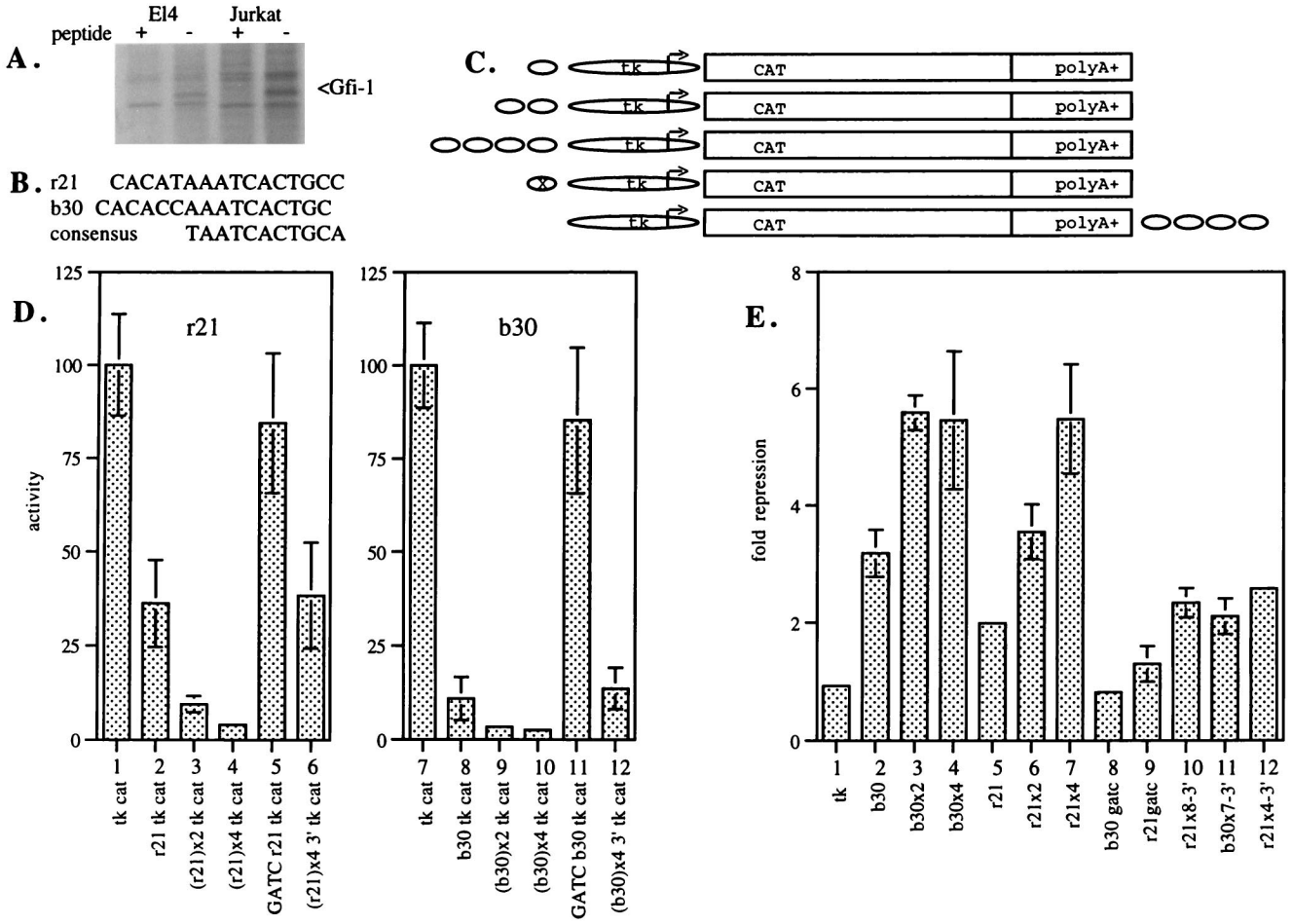

FIG. 1. Gfi-1 binding sites are *cis*-acting, position-independent repressor elements in Gfi-1-expressing T cells. (A) Gfi-1 antiserum was raised against a multiple-antigen peptide encoding amino acids 12 to 26 of rat Gfi-1 (anti-amino-terminus antibody; detailed in Materials and Methods). When affinity purified against a multiple-antigen peptide column, the antiserum immunoprecipitates a [^{35}S-labelled protein of approximately 60 kDa in extracts of EL4 and Jurkat T-cell lymphoma lines, which can be inhibited with the peptide immunogen. (B) Alignment of two high-affinity in vitro-selected Gfi-1 binding sites, r21 and b30, to the 12-bp consensus Gfi-1-binding site (38). (C) Oligonucleotides containing the sequence of r21 or b30 were used to construct a number of reporter constructs containing the bacterial CAT gene driven by the herpes simplex virus tk promoter with either one, two, or four sites or one GATC mutant (X) site 5′ or a number of sites 3′ of the tk-CAT cassette (indicated by small ovals). (D) The reporter constructs (5 μg) were cotransfected by electroporation into EL4 or Jurkat cells with a mutant CMV MIE-promoter-driven β-galactosidase construct (1 μg), which is not responsive to Gfi-1 repression (mutant B) (38). For electroporation, each transfection was done six times in each experiment. Extracts were assayed for β-galactosidase activity and normalized for transfection efficiency, and the normalized extracts were then assayed for CAT activity. The results were standardized to tk-CAT levels, which were set to 100. Standard deviation, depicted by error bars, is not shown when smaller than 1.8. (E) The reporter constructs (1.8 μg) were cotransfected by Lipofectamine into NIH 3T3 cells with the CMV MIE–β-galactosidase (50 ng) and either a Gfi-1 expression vector (10 ng) or an empty CMV5 vector plasmid (10 ng). The activity of the reporter construct with the CMV5 plasmid was divided by the activity of the reporter cotransfected with 10 ng of Gfi-1 expression construct and expressed in terms of fold repression. Standard deviation, depicted by error bars, is not shown when smaller than 1.8.

AATC core of either oligonucleotide was mutated to GATC (Fig. 1D, lanes 5 and 11). Since the GATC mutation reduces Gfi-1 binding by 95% in vitro (38), these data suggest that repression is likely to be dependent on Gfi-1 binding.

To confirm that the repression of the r21-tk and b30-tk promoters in EL4 and Jurkat cells was Gfi-1 dependent, the reporter constructs were also transfected into NIH 3T3 cells, which do not express Gfi-1 (data not shown). The results showed that neither the r21 nor the b30 oligonucleotide repressed the activity of the tk promoter in these cells (data not shown). However, when a Gfi-1 expression construct was cotransfected, the reporter constructs with r21 or b30 oligonucleotides were repressed. Moreover, the pattern of repression was identical to that observed in Jurkat and EL4 cells (Fig. 1E). RNase protection analysis revealed that transcripts from Gfi-1-repressed promoters were less abundant than transcripts from nonrepressed promoters and that both Gfi-1-repressed and nonrepressed promoters initiated transcription from the same site (data not shown). Taken together, these data indicate that Gfi-1, endogenous or transfected, is an active transcriptional repressor protein which is able to function at a distance.

DNA binding is necessary for Gfi-1 repression. Mutation of the AATC core of the b30 and r21 oligonucleotides to GATC resulted in loss of repression of the r21 tk-CAT and b30 tk-CAT reporter constructs in Jurkat and EL4 cells (Fig. 1D). The same loss of repression was also observed when the GATC mutant reporter constructs were transfected with or without Gfi-1 into NIH 3T3 cells (Fig. 1E, lanes 8 and 9). To confirm this result, we examined whether mutants of Gfi-1 that fail to bind the Gfi-1-binding motif continue to exhibit repressor activity. Therefore, a series of Gfi-1 zinc finger deletion mutants in which each of the zinc fingers were deleted one at a time (ΔZN1 to ΔZN6) or all together (ΔZN) (Fig. 2A) were con-

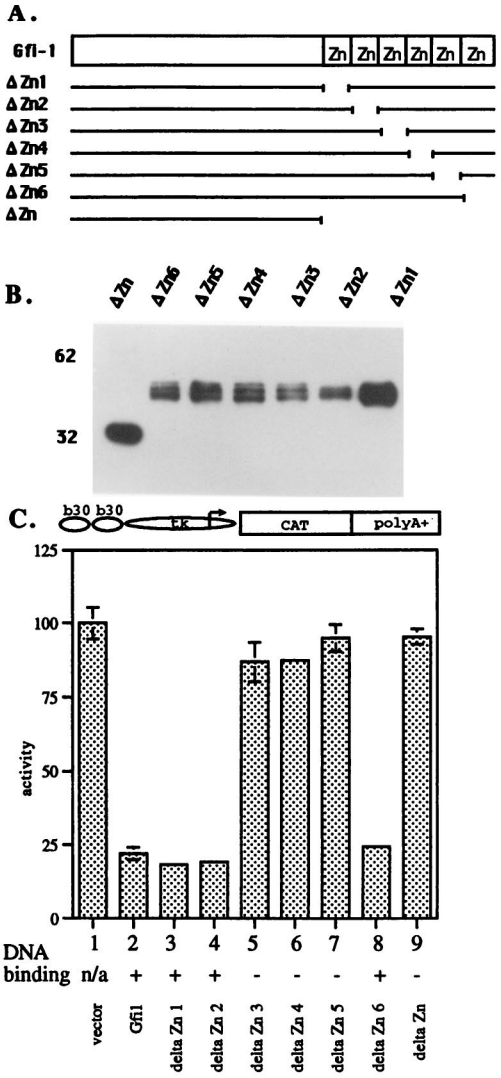

FIG. 2. DNA binding is necessary for Gfi-1 repression. (A) Line diagram of the series of deletion mutants of the Gfi-1 zinc fingers, constructed by overlap extension PCR. (B) Expression constructs encoding the mutants were transfected into NIH 3T3 cells, and extracts of the transfected cells were assayed by Western blotting with the anti-amino-terminal antibody. (C) A reporter construct in which two b30 sites were cloned 5′ of the tk-CAT cassette (2×b30 tk-CAT [Fig. 1]) was cotransfected with the CMV MIE–β-galactosidase plasmid, and either mutant expression constructs or an empty CMV5 vector plasmid. Extracts of transfected cells were assayed for β-galactosidase activity and normalized for transfection efficiency, and the normalized extracts were then assayed for CAT activity. Results were standardized to tk-CAT levels cotransfected with CMV5, which were set to 100. Standard deviation, depicted by error bars, is not shown when smaller than 1.8. DNA binding of the mutants was performed as reported previously (38).

structed. Our earlier studies had shown that deletion of zinc finger 3, 4, or 5 abolished DNA binding in vitro (38). When transfected into NIH 3T3 cells, mutant proteins accumulate to approximately equal levels (Fig. 2B) and are localized to the nucleus (data not shown). When cotransfected with (2×b30) tk-CAT reporter constructs into NIH 3T3 cells, the results showed that only mutants binding DNA were able to repress (Fig. 2C) and that expression of the mutants defective in DNA binding was not toxic in NIH 3T3 cells. Since the Gfi-1-mediated repression depends on DNA binding, it is unlikely to be due to squelching (the nonspecific titration of factors necessary for basal or induced transcription) (14).

Gfi-1 contains a modular repressor domain. The preceding data suggested that Gfi-1 is an active transcriptional repressor. Since such repressors are modular, we examined whether Gfi-1 contains a domain which confers transcriptional repression activity upon fusion to a heterologous DNA-binding protein. The amino-terminal non-zinc-finger region of Gfi-1 (amino acids 1 to 258) was fused to the amino terminus of a bacterial DNA-binding protein, LexA (8) (Fig. 3A). Cotransfection of LexA or Gfi-1–LexA fusion constructs with tk-CAT or Lex operator tk-CAT reporter constructs into NIH 3T3 cells revealed that while LexA represses neither reporter (Fig. 3B, lanes 2 and 5), Gfi-1–LexA represses only the Lex operator tk-CAT reporter (Fig. 3B, lanes 3 and 6). Therefore, the amino terminus of Gfi-1 transforms LexA into a sequence-specific active repressor protein and demonstrates the presence of a repressor domain in the amino terminus of Gfi-1.

The N-terminal 20 amino acids of Gfi-1 constitutes a domain that is necessary for repression. To map the repressor domain within Gfi-1, we constructed a series of amino-terminal deletion mutants (Fig. 4A). Since the amino-terminal 20 amino acids of the Gfi-1 protein contains a nuclear localization motif (see Fig. 5B), all the mutants were designed to contain this sequence. To address the potential role of these 20 amino acids in Gfi-1 repression function, two additional constructs were generated. In one (SV40 swap), the amino-terminal 20 amino acids were replaced by the SV40 NLS (21), whereas in the other (SV40 ZN), the SV40 NLS was fused to the amino terminus of the Gfi-1 zinc finger region (amino acids 252 to 308). Transient transfection of expression constructs of these mutants into NIH 3T3 cells followed by Western blotting re-

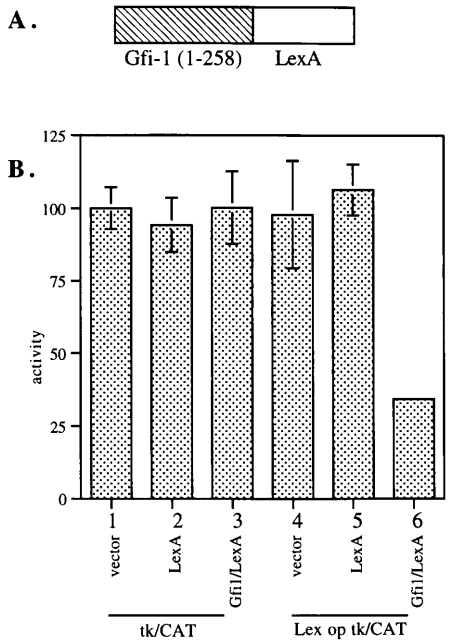

FIG. 3. The amino terminus of Gfi-1 transforms LexA into an active repressor. (A) Diagram of the construct generated by fusion of the first 258 amino acids of Gfi-1 to the amino terminus of the bacterial LexA protein. (B) Reporter constructs tk-CAT or Lex-op tk-CAT, which lack or contain, respectively, an oligonucleotide encoding a LexA-binding site, were cotransfected with the CMV MIE–β-galactosidase plasmid and either the empty CMV5 vector or expression vectors encoding LexA, or the Gfi-1–LexA fusion protein. Extracts of transfected cells were assayed for β-galactosidase activity and normalized for transfection efficiency, and the normalized extracts were assayed for CAT activity. Results were standardized to tk-CAT levels cotransfected with CMV5, which were set to 100. Standard deviation, depicted by error bars, is not shown when smaller than 1.8.

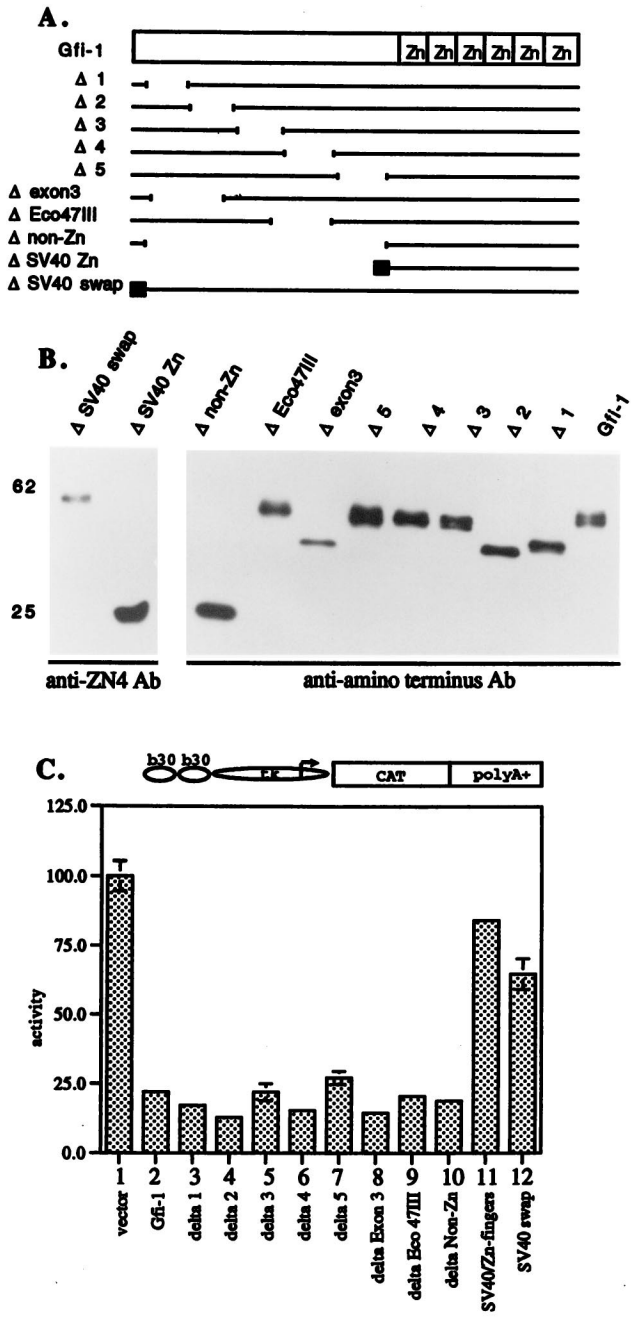

FIG. 4. The amino-terminal 20 amino acids of Gfi-1 is necessary for repression. (A) A diagram of the series of deletion mutants of the Gfi-1 amino terminus, constructed by overlap extension PCR. Solid boxes denote the sequence MGAPPKKKRKVA, which is the nuclear localization motif of the SV40 large T antigen (SV40 NLS) (21). (B) Expression constructs encoding the mutants were transfected into NIH 3T3 cells, and extracts of the transfected cells were assayed by Western blotting with the anti-ZN4 antibody (Ab) (antiserum raised against amino acids 336 to 350 of Gfi-1) or the anti-amino-terminus antibody. (C) A (2×b30)tk/CAT reporter construct was cotransfected with the CMV MIE–β-galactosidase plasmid and either mutant expression constructs or an empty CMV5 vector plasmid. Extracts of transfected cells were assayed for β-galactosidase activity and normalized for transfection efficiency, and the normalized extracts were assayed for CAT activity. Results were standardized to tk-CAT levels cotransfected with CMV5, which were set to 100. Standard deviation, depicted by error bars, is not shown when smaller than 1.8.

vealed that all the mutant proteins accumulated to approximately equal levels, with the exception of mutant Δexon 3, which lacks the entire exon 3 and mutants Δ1 and Δ2, which lack portions of the same exon (Fig. 4B). On longer exposure, a small amount of full-length Δexon 3 and Δ1 and Δ2 proteins were detected, suggesting that these proteins are synthesized but are relatively unstable. Therefore, exon 3 may be required for protein stability.

Transfection of these mutants, or wild-type Gfi-1, into NIH 3T3 cells revealed that all the proteins containing Gfi-1 amino acids 1 through 20 were able to repress the (2×b30) tk/CAT but not the tk-CAT reporter construct (Fig. 4C, and data not shown). Particularly interesting were the Δnon-ZN construct, which contains only the first 20 amino acids of Gfi-1 linked to the zinc fingers and represses as well as the full-length protein (Fig. 4C, lanes 2 and 10), and the SV40 ZN and SV40 swap mutants, which lack the first 20 amino acids and exhibit a severe loss of repressor activity (Fig. 4C, lanes 11 and 12). Therefore, the first 20 amino acids of Gfi-1 constitutes a domain that is necessary for Gfi-1-mediated transcriptional repression. The identification of the repressor domain and the ability of Gfi-1 to repress multiple promoters (Fig. 1 and reference 38) make it unlikely that repression by Gfi-1 will be limited by promoter context.

The Gfi-1 repressor domain, SNAG, defines an evolutionarily conserved family of transcriptional repressors. To determine whether the 20-amino-acid repressor domain of Gfi-1 is evolutionarily conserved, we cloned the Gfi-1 human homolog from a Jurkat cell cDNA library and the murine homolog from a genomic library. Sequence analysis of the human and murine clone and comparison with the rat clone revealed 100% identity in this but not other regions of the protein (Fig. 5A).

Gfi-1 is a member of a gene family. Using a *Gfi*-1 zinc finger DNA probe and low-stringency hybridization, we cloned a Gfi-1-related gene (*Gfi*-1B) which is 97% identical to *Gfi*-1 in the zinc finger domain. Comparison of Gfi-1 with Gfi-1B in the N-terminal non-zinc-finger region, however, revealed complete divergence with the exception of the 20-amino-acid repressor domain, in which the two proteins were 90% identical (Fig. 5A). Gfi-1B expression is restricted to lymphoid tissues, but it is not expressed in T cells. As expected, Gfi-1B binds the same DNA sequence as Gfi-1 and functions in a manner similar to Gfi-1 as an active transcriptional repressor (37a).

To determine whether the Gfi-1 repressor domain is shared between the members of the Gfi-1 family of transcriptional repressors and other transcriptional regulators, we screened the available sequence databases for proteins containing homologous sequences. This detected sequences with a high degree of homology to the Gfi-1 repressor domain in all of the vertebrate but not the *Drosophila* members of the Snail/Slug family of zinc finger proteins (Snail/Gfi-1, SNAG domain); the insulinoma-associated zinc finger protein IA-1, which is induced during the progression stage of human insulinomas and other neuroendocrine neoplasms including small cell lung carcinomas (23); and the homeobox protein Gsh-1, which is involved in pituitary development (26) (Fig. 5A). Although the functional role of the SNAG-related sequences in these proteins has not been determined, their homology to the SNAG domain suggests that they also function as transcriptional repressors. These findings suggest that the novel repression mechanism mediated by SNAG domains is evolutionarily conserved and active in multiple cell lineages.

Mutational analysis of the SNAG domain defines its role in directing Gfi-1 to the nucleus and in mediating transcriptional repression. To confirm that the SNAG domain contributes to the nuclear localization of Gfi-1, we carried out a mutational

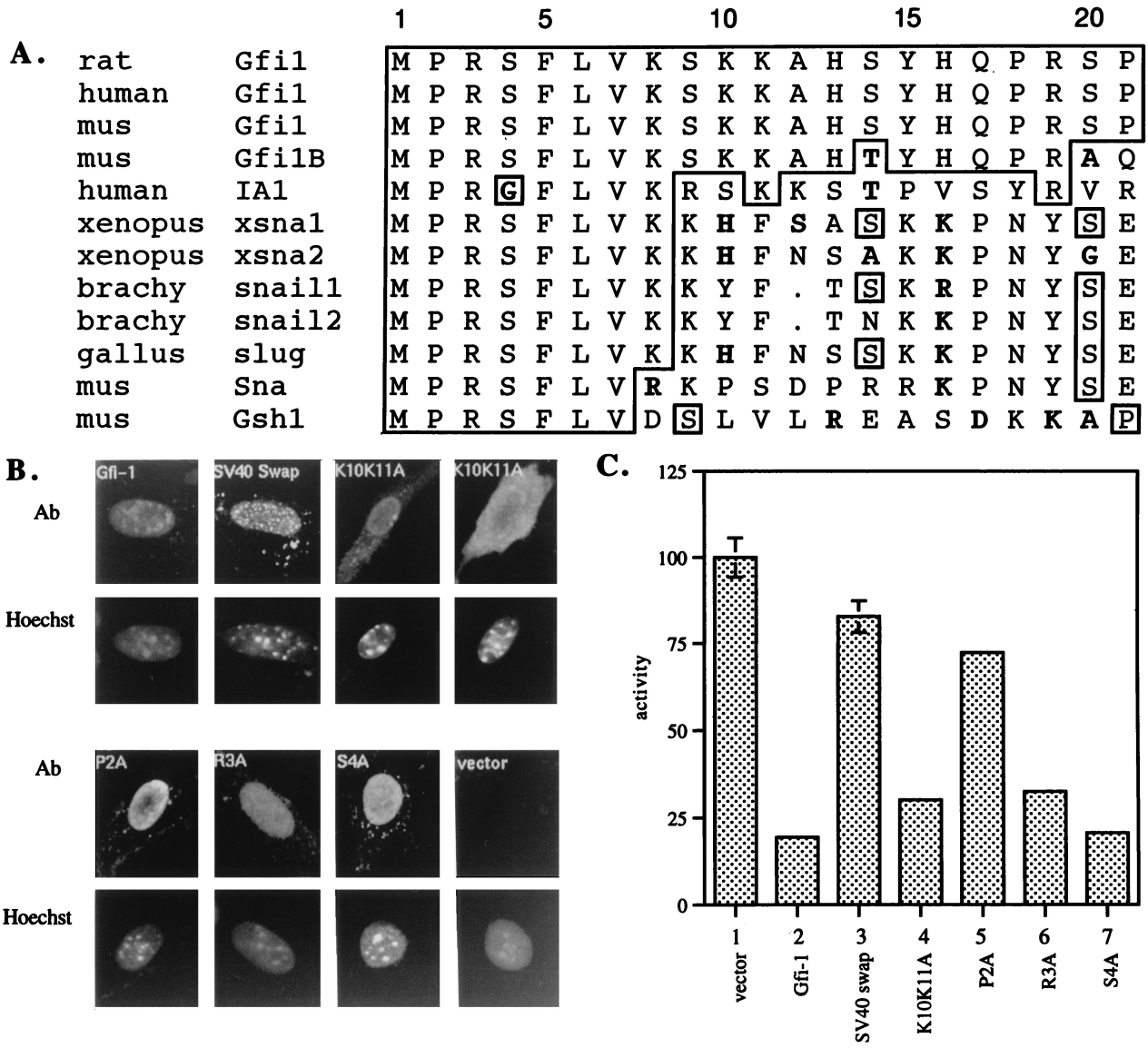

FIG. 5. The Gfi-1 repression domain is evolutionarily conserved, defines a family of repressors, and also encodes a nuclear localization motif. (A) Protein sequences from the amino termini of several gene products found in the GenBank database by the BLAST program (1) (with expected values set to 1,000) were aligned with Gfi-1 sequences for the human Gfi-1 and the Gfi-1-related gene Gfi-1B. The GenBank accession numbers for the sequences are as follows: rat Gfi-1, L06986; human and *Mus* Gfi-1, unpublished; *Mus* Gfi-1B, unpublished; human IA-1, U07172; *Xenopus* xsna1, X53450; *Xenopus* xsna2, X80269; *Brachydanio* (brachy) snail1, S68799; *Brachydanio* (brachy) snail2, U24225; *Gallus* slug, X77572; *Mus* Sna, X67253; *Mus* Gsh-1, U21224. Conservative amino acid substitutions are indicated in boldface type. (B) A series of single amino acid alanine substitution mutants with mutations in the repressor domain were constructed by overlap extension PCR. Expression constructs encoding the mutants were transfected into NIH 3T3 cells, immunofluorescence was performed with the affinity-purified anti-amino-terminus antibody (Ab), and the cells were counterstained with the DNA intercalation dye Hoechst 33258. The results were analyzed by confocal microscopy, and representative pictures are shown. (C) A (2×b30)tk/CAT reporter construct was cotransfected with the CMV MIE–β-galactosidase plasmid and either mutant expression constructs or an empty CMV5 vector plasmid. Extracts of transfected cells were assayed for β-galactosidase activity and normalized for transfection efficiency, and the normalized extracts were assayed for CAT activity. Results were standardized to tk-CAT levels cotransfected with CMV5, which were set to 100. Standard deviation, depicted by error bars, is not shown when smaller than 1.8.

analysis of this domain. In this analysis, we first mutated the lysines of the putative NLS at positions 10 and 11 into alanine (K10K11A) and examined the subcellular localization and repressor activity of the protein. Confocal immunofluorescence microscopy of the transfected cultures revealed that although Gfi-1 is strictly nuclear, the mutant protein is distributed equally to all subcellular compartments in approximately half of the K10K11A-expressing cells (Fig. 5B). In the remaining half of these cells, the protein is primarily although not totally nuclear (Fig. 5B). This result indicates that the putative NLS within the Gfi-1 SNAG domain plays a significant role in directing the protein to the nucleus. These changes in the subcellular localization of the protein were associated with modest abrogation of transcriptional repression (Fig. 5C, lane 4).

Subsequently, we mutated the proline at position 2 and the arginine at position 3 into alanine (P2A and R3A). These two amino acids were selected because they are conserved among all the currently known SNAG domains. In addition, we mutated the non-fully conserved serine at position 4 into alanine (S4A). Of these mutants, P2A was almost completely inactive

as a repressor (Fig. 5C, lane 5), while the R3A and S4A mutants were either partially or completely active as repressors (lanes 6 and 7). Thus, a single amino acid substitution (in mutant P2A) was able to impair the repressor function of the entire protein, confirming the presence of a repressor domain in the amino terminus of the protein. The subcellular localization of all these mutants was nuclear (Fig. 5B). Therefore, the effects of these mutations on the repressor activity of the SNAG domain were not due to changes in the subcellular localization of the protein.

The Gfi-1 SNAG domain depends on a titratable associated protein(s) for function. Active repressor domains inhibit transcription by interacting with transcriptional activators or the basal transcriptional complex either directly or indirectly through repressor adaptor proteins (reviewed in reference 20). When expressed as modules, separated from their DNA-binding domain, they interfere with the function of the DNA-bound cognate protein by titrating out interacting proteins necessary for transcription regulation (3, 30). To determine whether the Gfi-1 repressor function depends on titratable interacting proteins, we cotransfected the wild-type Gfi-1 expression construct and the (2×b30) tk-CAT reporter with increasing concentrations of the ΔZN expression construct (Fig. 6A) into EL4 and Jurkat cells. Transfection resulted in nonsequence-specific repression of the tk-CAT, CMV MIE (mutant B [38]) or SV40-CAT reporter constructs (data not shown), suggesting that expression of ΔZN in these cells is toxic. To avoid the complications engendered by toxicity, we performed the assay with NIH 3T3 cells. The results showed that the ΔZN construct interfered with the Gfi-1-mediated repression of the (2×b30) tk-CAT reporter in a dose-dependent manner (Fig. 6B, lanes 3 through 6), while the amino terminus of the SV40 swap mutant, which lacks the repressor domain, failed to inhibit the Gfi-1 mediated repression (Fig. 6B, lane 7). Immunofluorescence of NIH 3T3 cells transfected with the ΔZN or the SV40 ΔZN construct revealed that while the SV40 ΔZN protein is strictly nuclear, the ΔZN protein is distributed equally between the nucleus and the cytoplasm (Fig. 6C). Therefore, the difference between two proteins in their ability to inhibit the Gfi-1-mediated repression is likely to be underestimated by this analysis, since the intranuclear concentration of the SV40 SWAP ΔZN mutant should be much greater than that of the ΔZN. We conclude that the delta ZN construct functions as a dominant negative mutant by binding to titratable proteins which are necessary for Gfi-1 repressor domain function.

Gfi-1 releases cells from a G_1 arrest induced by IL-2 withdrawal. A subline (2780a) of the IL-2-dependent T-cell lymphoma line 2780, from which Gfi-1 was originally cloned, was infected with the SRα retrovirus vector or with an SRα–Gfi-1 construct. Three independent SRα–Gfi-1 infections gave rise to three independently maintained cell lines, all overexpressing Gfi-1 (Fig. 7). To determine whether overexpression of Gfi-1 affected cell viability following IL-2 withdrawal, the following experiment was carried out. A total of 10^7 cells from each infected culture were placed in 10 ml of IL-2 (100 U/ml)-containing medium. One day later, while the cells were growing logarithmically, they were harvested, and subcultures of 10^6 cells per ml in IL-2-deficient medium were made in triplicate. Live cells in each subculture were counted daily for 96 h. The results showed a significantly greater number of live cells in the Gfi-1-expressing cultures than in the empty vector-infected cultures (Table 1). In parallel with the cell counting, cultures were harvested at 24, 48, and 72 h following IL-2 withdrawal and their cell cycle distribution was determined by fluorescence-activated cell sorter analysis. The results showed that at

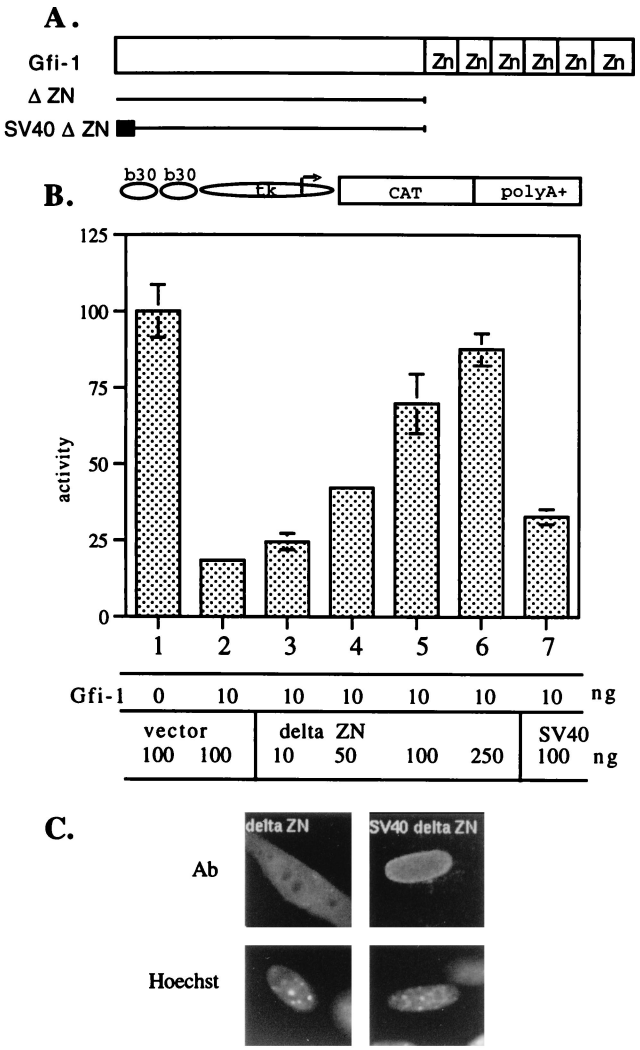

FIG. 6. The Gfi-1 SNAG domain depends on a titratable associated protein(s) for function. (A) Diagram of mutants of Gfi-1 which encode either the first 258 amino acids of Gfi-1 (ΔZN), or the SV40 NLS fused to amino acids 21 to 258 of Gfi-1 (SV40 ΔZN). (B) A (2×b30)tk/CAT reporter construct was cotransfected with the CMV MIE–β-galactosidase plasmid, and either an empty CMV5 vector plasmid (lane 1) or 10 ng of Gfi-1 expression construct (lanes 2 to 7). In addition, 100 ng of CMV5 vector plasmid (lanes 1 and 2), 10 ng to 250 ng of the ΔZN construct (lanes 3 to 6), or 100 ng of SV40 ΔZN construct (lane 7) were cotransfected. Extracts of transfected cells were assayed for β-galactosidase activity and normalized for transfection efficiency, and the normalized extracts were assayed for CAT activity. Results were standardized to tk-CAT levels cotransfected with CMV5, which were set to 100. (C) Expression constructs encoding the mutants were transfected into NIH 3T3 cells, immunofluorescence was performed with the affinity-purified anti-amino-terminus antibody (Ab), and the cells were counterstained with the DNA intercalation dye Hoechst 33258. The results were analyzed by confocal microscopy, and representative pictures are shown.

24 and 48 h, both the SRα- and the SRα–Gfi-1-infected cultures went into G_1 arrest. However, at 72 h following IL-2 withdrawal, the SRα–Gfi-1-expressing cells began to escape from the G_1 arrest and to reenter the cell cycle (Table 2).

To determine whether the ability of Gfi-1 to inhibit the G_1 arrest induced by IL-2 withdrawal was dependent upon the Gfi-1 repression function, Gfi-1 and the P2A–Gfi-1 mutant were introduced into 2780 cells by using SRα retrovirus constructs. Following IL-2 withdrawal, cells expressing the P2A mutant, which is defective in transcription function, failed to

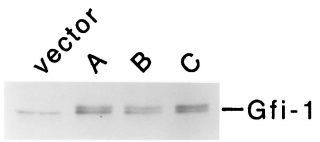

FIG. 7. Gfi-1 is overexpressed in 2780a cells infected with the SRα–Gfi-1 virus. 2780a cells were infected with the SRα–Gfi-1 virus construct and selected for G418 resistance. Lysates from three independent mass cultures (A, B, and C) of G418-resistant empty-vector, or SRα–Gfi-1-infected cells were analyzed by Western blotting with the anti-amino-terminus antibody.

escape from G_1 arrest and were indistinguishable from the SRα-infected control cultures (Table 3). Therefore, the Gfi-1 mediated inhibition G_1 arrest in 2780 cells grown in the absence of IL-2 depends on the Gfi-1 SNAG repression function.

DISCUSSION

In this report, we present evidence showing that Gfi-1 is a position- and orientation-independent active transcriptional repressor. The Gfi-1 transcriptional repression is mediated by a transferable repressor domain which has been mapped to the first 20 amino acids at the N terminus of the protein. This novel domain is evolutionarily conserved and identifies a family of repressor proteins. Mutations of critical amino acids within this domain can severely inhibit transcriptional repression and nuclear localization functions of the Gfi-1 protein.

Transcriptional repression is mediated by a variety of molecular mechanisms. These include (i) the induction of a repressed chromatin state; (ii) the direct competition between transcriptional repressors and activators for the same DNA-binding site; (iii) the repressor-mediated masking of the ability of an activator to contact the transcriptional complex, also called quenching or short-range repression; and (iv) the repressor-dependent hindrance in the formation of the transcriptional complex. The last two mechanisms are mediated by transferable repressor domains that link the repressor with an activator (quenching) or the basic transcriptional complex (active repression), either directly or via corepressor proteins (reviewed in reference 20). The data in this report demonstrate that Gfi-1 contains a transferable repressor domain that functions at a distance and represses multiple promoters including the minimal tk, the CMV MIE, and SV40 early promoters. These data place Gfi-1 into the group of active transcriptional repressors. Transferable repressor domains have been detected in a limited number of transcription factors, including the *Drosophila* engrailed (en) (17), even-skipped (*eve*) (19) and Krüppel (Kr) (28) proteins; the human YY1 (37) and other mammalian Krüppel-related proteins; the tumor suppressor protein WT1 (31); the human bZIP factor E4BP4 (10); the mouse b-HLH-Zip factor Mxi-1 (35); and the thyroid and retinoic acid receptors (4). The primary amino acid sequences of these repressor domains are dissimilar. Moreover, none of them exhibits any similarity to the repressor domain of Gfi-1 described in this report. This suggests that each repressor domain may interact with a different set of proteins. Although some of the repressor domains such as the KRAB domain of Krüppel and Krüppel-like proteins contain characteristic motifs (32), the repressor domain of Gfi-1 is highly charged but exhibits no characteristic structure.

The Gfi-1 repressor domain is shared by Gfi-1, Gfi-1B (a Gfi-1-related protein), the vertebrate members of the Snail-Slug family of proteins, IA-1 (a poorly characterized zinc finger protein expressed in a variety of human neoplasms of neuroendocrine origin including insulinomas and small cell lung carcinomas) (23), and the Hox protein Gsh-1 (expressed solely in neural tissues) (26). This suggests that Gfi-1 may identify a mechanism of transcriptional repression that is conserved through evolution and is shared by a variety of cell types.

The snail protein originally identified in *Drosophila melanogaster* is induced by dorsal (dl) in the mesodermal anlage and functions to maintain proper germ layer boundaries by repressing the expression of regulatory genes of the lateral mesecto-

TABLE 1. Inhibition by Gfi-1 of cell death induced by IL-2 withdrawal

Time (h)	Live-cell counts[a] (10^3) (mean ± SD)			
	SRα	Gfi-1A	Gfi-1B	Gfi-1C
24	1,300 ± 32	1,240 ± 74	1,366 ± 124	1,374 ± 116
48	1,146 ± 68	1,234 ± 80	1,206 ± 50	1,226 ± 148
72	598 ± 184	834 ± 132	954 ± 24	1,020 ± 100
96	480 ± 134	780 ± 118	940 ± 58	874 ± 34

[a] IL-2-dependent 2780a cells infected with empty vector or SRα–Gfi-1 retroviruses (Western blot analysis in Fig. 7) were placed in culture with 100 U of IL-2. At 24 h after addition of IL-2, the cells were centrifuged out of the IL-2-containing medium, washed twice, and replated in triplicate cultures of 10^6 cells per ml in IL-2-deficient medium. Live cells in each subculture were counted daily for 96 h.

TABLE 2. Escape from a G_1 checkpoint induced by IL-2 withdrawal

Infection[b]	% of cells at stages of cell cycle at[b]:					
	24 h			72 h		
	G_1[b]	S	G_2/M	G_1	S	G_2/M
SRα A	87	6	7	96	3	1
SRα B	89	6	5	97	3	0
SRα C	85	8	7	96	3	1
Gfi-1 A	88	5	8	78	17	5
Gfi-1 B	86	8	6	75	18	7
Gfi-1 C	88	6	6	81	14	5

[a] IL-2-dependent 2780a cells infected with empty vector or SRα–Gfi-1 retroviruses were IL-2 starved as described for Table 1. A, B, and C refer to independently infected sublines of 2780a cells.

[b] Samples were removed from the culture daily and stained for DNA analysis by resuspending pellets in FACS buffer (36). The relative distribution within the cell cycle was determined with the MacCycle program (Phoenix Flow Systems, Inc.).

TABLE 3. SNAG dependence of Gfi-1-mediated escape from G_1 arrest

Infection[a]	% of cells at following stage of cell cycle after 72 h[b]:		
	G_1	S	G_2/M
SRα A	95.2	3.4	1.2
Gfi-1 A	80.8	16.0	3.2
Gfi-1 B	87.7	10.0	2.1
Gfi-1 C	74.5	20.8	4.6
P2A A	93.0	4.9	1.9
P2A B	93.9	4.5	1.4
P2A C	96.9	2.0	0.9

[a] IL-2-dependent 2780a cells infected with empty vector, SRα–Gfi-1, or SRα–P2A retroviruses were IL-2 starved as described for Table 1. A, B, and C refer to independently infected sublines of 2780 cells.

[b] Samples were processed as described in Table 2, footnote *b*.

derm and neuroectoderm within the mesoderm (22, 25). The most striking difference between *Drosophila* snail and its vertebrate homologs is that the latter contain a Gfi-1-like repressor domain at their N termini while the *Drosophila* protein does not. *Drosophila* snail quenches transcriptional activators bound to promoter DNA at a distance shorter than 150 bp from the site of snail binding, permitting separate enhancers to function autonomously within a complex promoter (15). In contrast, vertebrate homologs of snail may combine short-range repression with Gfi-1-like long-range repression. This would mean that *Drosophila* snail and its vertebrate homologs may contribute to development by different repression mechanisms.

Mutational analysis of the Gfi-1 SNAG domain revealed that an NLS is coincident with the repressor domain. This signal, however, was not sufficient to cause the amino terminus of Gfi-1 (ΔZN) to be localized solely in the nucleus (Fig. 6C), suggesting that an additional NLS is present in the zinc finger domain. Deletion of individual zinc fingers failed to localize this signal to any one zinc finger. This finding is not without precedent, because mutational analysis of the NGFI-A (Egr-1) protein revealed that NGFI-A contains a novel bipartite NLS which is dependent on both the overall structure of the zinc finger DNA-binding domain and basic flanking sequences (33). Therefore, the nuclear localization of the Gfi-1 protein may be achieved by the concerted effect of an NLS within the SNAG domain and a secondary signal generated by the structure of the zinc finger domain.

The identification of *Gfi*-1 as a gene whose expression is induced by provirus integration during the selection of IL-2 dependent T-cell lymphoma lines for IL-2 independence suggested that *Gfi*-1 may promote T-cell growth in the absence of IL-2 (13). The results presented in this report confirmed that Gfi-1 contributes to the progression of T-cell lymphoma lines to IL-2 independence by abrogation of the G_1 arrest induced by IL-2 withdrawal. Withdrawal of IL-2 from cultures of the IL-2-dependent T-cell lymphoma line 2780 infected with SRα or SRα–Gfi-1 retroviruses induced G_1 arrest. However, the cells infected with the SRα–Gfi-1 retrovirus began to escape the arrest and to reenter the cell cycle at about 72 h following IL-2 withdrawal. The repression function of Gfi-1 and the inhibition of G_1 arrest appear to be causally linked. A P2A mutant of Gfi-1 which is defective as a repressor fails to release 2780 cells from G_1 arrest induced by IL-2 withdrawal.

Additional studies of the role of *Gfi*-1 and other oncogenes in leukemogenesis provide important hints on genes that may contribute to Gfi-1 regulation or may be regulated by Gfi-1. Thus, it has been shown that *Gfi*-1/*pal*-1 cooperates with *pim*-1 and c-*myc* in MoMuLV-inoculated Eμ/*pim*-1 and Eμ/c-*myc* transgenic mice. However, the activation of *Gfi*-1 and that of *Bmi*-1 appear to be mutually exclusive in these tumors (6). Bmi-1 is a member of the polycomb group of proteins and, like Gfi-1, functions as a transcriptional repressor when tethered to DNA (9). Therefore, the mutual exclusion of *Gfi*-1 and *Bmi*-1 activation may mean either that both repress the same targets or that the targets of either protein when repressed result in the same biological phenotype. On the other hand, activation of *Gfi*-1 or *Bmi*-1 in tumors carrying an activated *pim*-1 or c-*myc* gene suggest that these genes are functionally linked.

The studies presented in this report identify a new family transcriptional repressors, conserved through evolution and functional in different cell types. One member of this family, Gfi-1, releases cells from a G_1 block induced by IL-2 withdrawal and is targeted by proviral integration in a number of tumor systems.

ACKNOWLEDGMENTS

We thank Robin Muise for RNase protection analysis; Sam Litwin for assistance with statistical analysis; Marija Helt for confocal microscopy; Randy Strich, James Sherley, Erica Golemis, and the members of the Tsichlis laboratory for helpful discussions; and Pat Bateman for secretarial assistance.

This work was supported by Public Health Service grant CA56110. Additional support was provided by Public Health Service grant CA06927 and by an appropriation from the Commonwealth of Pennsylvania to the Fox Chase Cancer Center. H.L.G. is funded by NRSA fellowship CA59302.

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