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Schlafen, a New Family of Growth Regulatory Genes that Affect Thymocyte Development

David A. Schwarz,† Carol D. Katayama, and Stephen M. Hedrick*
Cancer Center and the Department of Biology
University of California, San Diego
La Jolla, California 92093-0687

Summary

The *Schlafen* (*Slfn*) family of genes are differentially regulated during thymocyte maturation and are preferentially expressed in the lymphoid tissues. Ectopic expression of the prototype member *Slfn1* early in the T lineage profoundly alters cell growth and development. In these mice, the DP thymocytes fail to complete maturation, and, depending on the transgene dosage, the number of thymocytes is reduced to 1%–30% of normal. Furthermore, expression of the *Schlafen* family members in fibroblasts and thymoma cells either retards or ablates cell growth. The conceptual protein sequences deduced for each of the family members have no similarity to characterized proteins and must therefore participate in a heretofore unknown regulatory mechanism guiding both cell growth and T cell development.

Introduction

The production of a functional T cell repertoire requires that each cell express a clonotypic T cell receptor (TCR) that is capable of engaging self-major histocompatibility complex (MHC) molecules and yet at the same time is not overtly autoreactive. The preponderance of evidence is consistent with the notion that a critical component of thymocyte selection is the overall affinity of each TCR for a combinatorial epitope composed of MHC bound to self-peptides (Ashton-Rickardt et al., 1994; Hogquist et al., 1994; Sebzda et al., 1994). Those thymocytes that lack a minimal affinity for this epitope are deduced to die from neglect, while thymocytes with too high an affinity are deleted from the repertoire—a process referred to as negative selection (Kappler et al., 1987). Thymocytes with an intermediate affinity receive an inductive stimulus, complete maturation, and become CD4 or CD8 single-positive (SP) T cells. This latter process is referred to as positive selection (Bevan, 1977; Zinkernagel et al., 1978).

Like their immature counterparts, peripheral T cells exhibit distinct biological responses as a consequence of the interaction between the TCR and its cognate MHC/peptide ligand, including clonal expansion, blast transformation, and cytokine secretion. However, recent data suggest that while naive T lymphocytes have a relatively long lifespan in the periphery, their subsistence is dependent upon the continual engagement of self-MHC molecules. Without this interaction, survival is severely compromised (Takeda et al., 1996; Rooke et al., 1997; Tanchot et al., 1997). This finding is consistent with the observation that in peripheral T cells, certain components associated with signal transduction such as the ζ chain of the T cell receptor complex and ZAP-70, exist in a phosphorylated state indicative of receptor engagement (van Oers et al., 1996).

Given that peripheral T cells exist in a resting state, the signals that mediate survival presumably do not initiate entry into the cell cycle. Similarly, the process of positive selection also occurs in the absence of proliferation (Lundberg and Shortman, 1994; Ernst et al., 1995). Since both processes are regulated via receptor ligation, an event that normally causes T cell activation and growth, how are these cells maintained in a quiescent state? In part, the solution to this problem may rest in the overall affinity of the interaction between the TCR and its cognate MHC/peptide ligand. Low-affinity interactions may be sufficient to drive both positive selection and T cell survival and yet not drive the cells to enter the cell cycle. However, evidence is accumulating to suggest that maintenance of the quiescent state is an active rather than a passive process. The first indication of this fact was uncovered by Pepperkok and colleagues when they discovered that fibroblast growth could be inhibited by microinjecting cells with RNA from resting T cells (Pepperkok et al., 1988). The genetic loci conferring this inhibitory effect have yet to be defined but are likely to include the recently identified transcription factor LKLF, which appears to be critical in establishing a quiescent state (Kuo et al., 1997).

Because of our interest in understanding the biochemical events that guide thymocyte maturation, we set out to isolate and characterize genes that are differentially expressed and transcriptionally upregulated as a result of the positive selection process. In so doing, we have isolated a new family of genes whose members are differentially regulated during thymocyte development. However, while the prototype member of this family is dramatically induced following positive selection, it does not appear to be required for the selection process. Rather, this gene family is a potent suppressor of cell growth, and we suspect that it may be yet another integral component of the machinery that helps maintain T cell quiescence.

Results

Identification and Classification of the *Schlafen* Gene Family

The analyses of thymocyte development in vivo have benefited from the development of TCR transgenic mice. Since virtually all T cells in these mice express the same clonotypic antigen receptor, the population is uniformly subjected to the processes of positive and negative selection. This property permits the manipulation of the selection process by altering the MHC molecules encountered during development. For instance, in mice

*To whom correspondence should be addressed (e-mail: shedrick@ucsd.edu).
†Present Address: Neurocrine Biosciences, Inc., 10555 Science Center Drive, San Diego, CA 92121-1102.

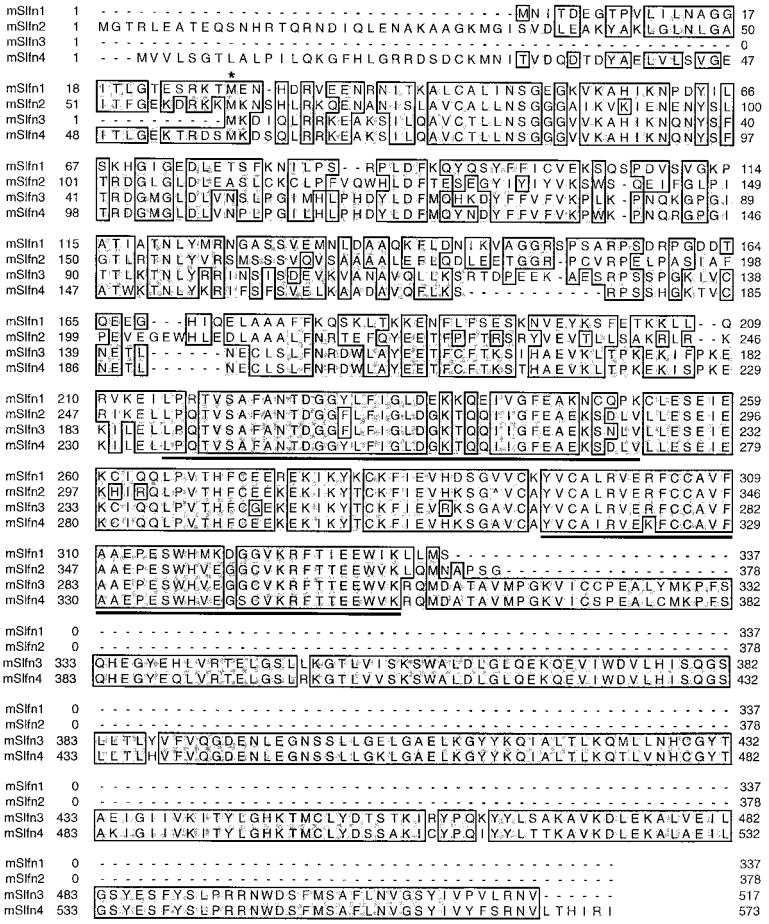

Figure 1. Comparison of the *Schlafen* Family Deduced Amino Acid Sequences

Comparison of the deduced amino acid sequences of the four complete *Schlafen* family genes. Conserved amino acids are boxed while identical sequences are shaded. The underlined regions define conserved sequence blocks identified using BlockMaker. The asterisk marks the location of the methionine residues that potentially could reside at the amino terminal end of each protein.

expressing the AND TCR specific for cytochrome *c*, and the H-2^{h4} haplotype (AND.4R mice), maturation is halted at the CD4^{+}8^{+} double-positive (DP) stage, and these mice lack mature CD4 or CD8 SP cells (Kersh and Hedrick, 1995). In contrast, development is skewed toward the positive selection of CD4 SP cells in AND mice expressing H-2^{b} (AND.B6 mice), and these cells constitute 50% of all thymocytes (Kaye et al., 1989, 1992).

To identify genes that are upregulated as a result of positive selection, a subtractive hybridization was performed using thymus cDNA libraries prepared from AND.B6 and AND.4R mice. The objective was to characterize genes from the AND.B6 library not in common with the AND.4R library. This led to the isolation of a novel developmentally regulated gene that is preferentially expressed in AND.B6 compared to AND.4R thymocytes (Figure 2A). The nucleotide and deduced amino acid sequences of this gene, which we have named *Schlafen1* (*Slfn1*), were used to query the expressed sequence tag (EST) databases using the gapped-basic local alignment search tool (BLAST) (Altschul et al., 1997). This approach ultimately led to the isolation of three additional *Slfn* genes (Figure 1) that collectively bear identity or similarity to 53 EST entries. Based on a sequence comparison, these ESTs have been assigned to six unique genes. Expression of each of these genes has been confirmed by rtPCR (below and data not shown). A portion of the *Slfn3* cDNA was also used to screen a mouse genomic library, resulting in the isolation of a series of clones that encode all or part of *Slfn3*, *4*, and *6*, as well as one additional clone that we have designated *Slfn7*. Based on these data, we estimate that the *Slfn* family consists of at least seven distinct genes.

Each *Slfn* cDNA encodes a single long open reading frame that is initiated by a consensus translational initiation codon (Kozak, 1986) and is preceded upstream by one or more in frame termination codons. In three of the genes (*Slfn1*, *2*, and *4*), an internal Kozak initiation codon is found corresponding to amino acid position 28 of SLFN1, and it is unclear which initiation codon is utilized by the mature protein; however, since the upstream methionine codon is missing from *Slfn3*, it is conceivable that all four proteins preferentially use this internal initiation site.

The four conceptual SLFN proteins contain a core region that is nearly identical, spanning amino acids 213–336 of SLFN1; however, the N terminus for each of the four proteins differs significantly. The exception is between SLFN3 and 4, which are very similar except for a deletion of 10 amino acids in SLFN4. Interestingly, while SLFN1 and 2 are almost identical in size, SLFN3 and 4 are nearly twice as large, possessing a carboxy-terminal protein sequence of approximately 200 amino acids not found in SLFN1 or 2. We refer to these regions, based on the amino acid sequence of SLFN3, as domain I (amino acids 1–186), II (amino acids 187–309), and III

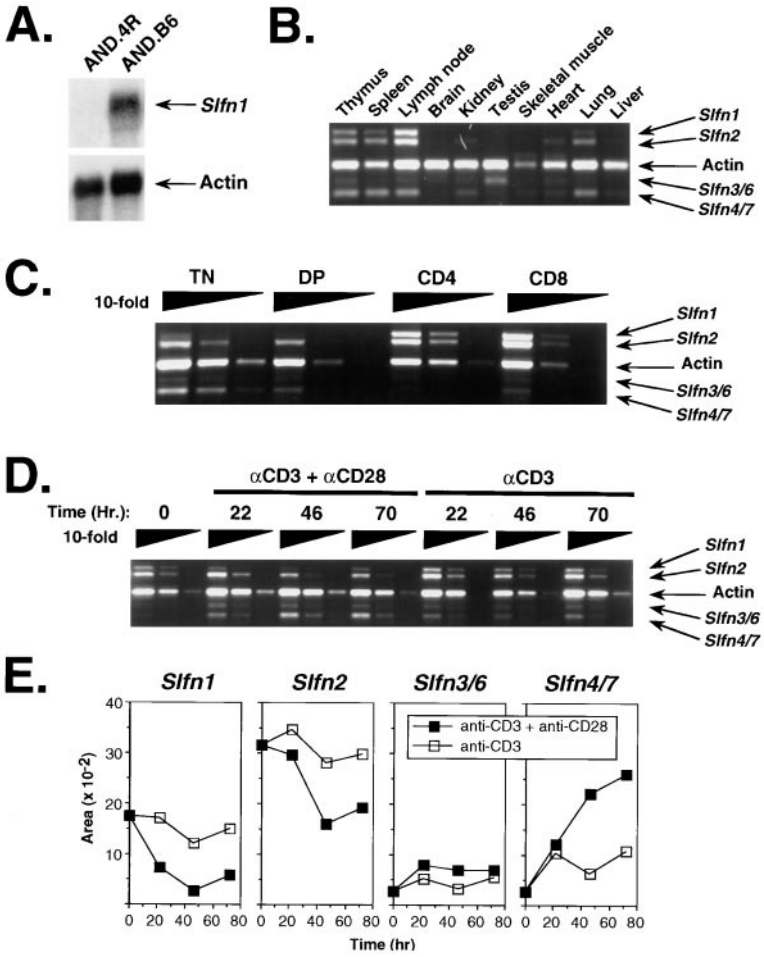

Figure 2. Analysis of **Schlafen** Messenger RNA Expression

(A) Total RNA was isolated from the thymi of AND mice and analyzed by Northern hybridization using radiolabeled *Slfn1* cDNA. Alternatively, rtPCR analysis was used to measure message expression in the indicated organs (B), thymic subsets (C), or resting versus activated T cells (D). These data are representative of two experiments that were performed using different primer cocktails. (E) The area encompassed by each PCR product in the undiluted cDNA time points from (D) was calculated, and the area of each *Slfn* gene was normalized relative to actin and plotted.

(amino acids 310–517). Although we have no information to indicate that these regions constitute actual protein domains, domain III of both SLFN3 and 4 are encoded by a unique exon, while domains I and II are contained within a single exon (data not shown).

Analysis of *Schlafen* Family Sequences

Using gapped-BLASTn to query the nonredundant DNA databases, we found that the nucleotide sequences of Slfn1-4 do not share meaningful similarities to any known genes. In contrast, a number of significant matches were found when the deduced SLFN amino acid sequences were used to query the nonredundant protein databases using BLASTp. In each case, strong matches were found in the right inverted terminal repeat (ITR) of Vaccinia, Variola, and Cowpox viruses. Located within this region are the putative proteins referred to as B2R, 20.9k, B3, H3R, and H5R. Upon closer examination, it was found that the nucleotide sequences of these viral isolates are very similar to those for the *Slfn* family; however, with the exception of B2R, the similarity is not found within a single ORF but rather is distributed through all three ORFs. We suspect that a member of the *Slfn* family was captured early on in the evolution of this viral family and has been slowly decaying over time. Nonetheless, there exists the possibility that one or more of the putative ORFs encode a functional protein that affects viral replication or host cell function. In the absence of nonviral homologs at either the nucleotide or protein level, the analysis of each family member was extended to identify any putative domain structures in each SLFN protein. The most recent releases of the PROSITE (Bairoch et al., 1997), BLOCKS (Henikoff and Henikoff, 1994), and PRINTS (Attwood et al., 1998) databases were queried. In no case were multiple members of a motif "family" present. We also used Block Maker (Henikoff et al., 1995) to identify two conserved motifs that were defined using SLFN1-4, along with the partial sequence for the human homolog of SLFN5. These motifs, which are underlined in Figure 1, were used to query the nonredundant protein databases using the local alignment of multiple alignments (LAMA) search tool (Pietrokovski, 1996). Again, as we saw using the individual sequences as query vectors, there were no significant matches found.

Expression of *Schlafen* Family Members

The *Slfn* genes are expressed at relatively low levels as detected by Northern blot analysis (Figure 2A; data not shown), so we have utilized rtPCR to measure the expression of each gene. Primer cocktails were used that target the nucleotide sequences encoding the variable domain I and conserved domain II of each gene. In this

way, we could accomplish the simultaneous amplification of all four genes in a single reaction. As a control, an additional primer set specific for β actin was included in all reactions. We note that the primer sets for *Slfn3* and *4* also amplify *Slfn6* and *7*, respectively.

As shown in Figure 2B, *Slfn1*, *2*, and *4/7* were found to be mainly expressed in the thymus, lymph node, and spleen. There was low-level expression in other tissues, especially the lung, but these samples probably contained circulating lymphocytes. The highest level of *Slfn3/6* expression was detected in the testis, although there was a small amount detected in the thymus and heart. Complement-mediated depletion of thy-1$^+$ splenocytes removed most of the *Slfn1* signal (data not shown), demonstrating that much of the expression can be attributed to the T cell subset. Conversely, we failed to find expression of *Slfn4/7* in purified T cells (Figure 2D), and presumably expression in the spleen and lymph nodes can be attributed to other cell types.

We next examined expression of each gene in the four major thymic subsets: immature CD3$^-$CD4$^-$CD8$^-$ triple-negative cells (TN), CD4$^+$CD8$^+$ DP cells, and mature CD4 or CD8 cells. In order to quantitate the relative expression in each subset, serial 10-fold dilutions of cDNA were PCR amplified, and all signals were normalized to β actin. As shown in Figure 2C, *Slfn1* was upregulated approximately 100-fold during the DP to SP transition. *Slfn2* expression also increased during this transition, but the increase was found to be only 5- to 10-fold. Interestingly, *Slfn4/7expression* decreased during development, being the highest in TN cells and barely detectable in SP cells. Finally, *Slfn3/6* message is expressed very weakly, but the level of its expression does not appear to change dramatically. Collectively, these data show that *Slfn1, 2, and 4/7* are preferentially expressed in the lymphoid lineage and are differentially regulated during thymocyte development.

We also examined the expression of *Slfn* genes under conditions of T cell activation. Peripheral T cells were purified and left untreated or subjected to plate-bound anti-CD3 in the presence or absence of CD28 costimulation for either 1, 2, or 3 days. Untreated T cells expressed both *Slfn1* and *2* but not *3/6 or 4/7* (Figure 2D). Following T cell activation, there was an increase in the expression of *Slfn3/6* and *4/7* and a concomitant decrease in the expression of *Slfn1* and *2* (Figure 2E). Interestingly, these changes were more pronounced following ligation of both CD3 and CD28. This may indicate that the expression of the *Slfn* gene family is linked to cell growth or blastogenesis.

Slfn Inhibits Cell Growth

Early attempts to produce cell lines that constitutively expressed *Slfn1* were unsuccessful. We also found that neither antigen-specific T cell clones nor primary T cell lineage transformed cell lines expressed *Slfn1* (data not shown). Since we had already found a tentative link between expression of the *Slfn* genes and cell growth (see above), we reasoned that ectopic expression of the *Slfn* family might alter normal cell growth. To test this hypothesis, cDNA clones for *Slfn1-3* were cloned into the expression vector pCIN-4 (Rees et al., 1996). This vector contains a ribosomal entry site that allows translation of both the gene of interest and the downstream neomycin phosphotransferase message. As a direct consequence, all transfected cells that are neomycin resistant must also express *Slfn*.

Figure 3A is a pictorial of three independent experiments using this expression system. In each case, transfection of NIH-3T6 cells with pCIN-4 alone resulted in innumerable drug resistant colonies, while no colonies could be detected in mock transfected cultures. However, cells transfected with pCIN-4 vectors encoding *Slfn* genes had a variable effect on the generation of drug-resistant colonies. With *Slfn1*, there was a noticeable reduction in the number of colonies recovered relative to vector alone. However, for *Slfn2* and *3*, there was a dramatic reduction in the number of colonies observed, with only a single colony produced in all three independent transfections with *Slfn3*. Similar results were also obtained using the thymoma AKR1G1 in a colony formation assay (data not shown). This presents an enigma, since AKR1 expresses a member of the *Slfn* family that is amplified by the *Slfn3* primer set (data not shown), and yet transfection appears to prevent colony formation. We are currently attempting to determine whether the detected transcript might encode a mutant form of *Slfn3* or whether our primers are detecting the closely related *Slfn6*, which we have yet to assay for growth disruption. Alternatively, colony formation may depend on the relative level of *Slfn3* expression.

The above data are consistent with the possibility that *Slfn* family members arrest cell growth, or cause apoptosis, or both. To account for the loss of *Slfn*-expressing cells, we placed *Slfn1* under control of the tetracycline operator (Shockett et al., 1995) and introduced this synthetic gene into NIH-3T3 fibroblasts expressing the tetracycline responsive transactivator (S2-6 cells, a generous gift from David Schatz). In these cells, expression of the transgene is suppressed in the presence of tetracycline and is induced following tetracycline removal. To test the effect of *Slfn1* on cell growth, expression was induced for periods of 24, 48, or 72 hr. In preliminary experiments, we found a strong inhibition of cell growth in the *Slfn1*-transfected clone but not in S2-6 clones. To determine the percentage of cells that were in G0/G1, S, and G2/M at different times after *Slfn1* induction, the cells were stained with propidium iodide to measure DNA content, and DNA synthesis was assessed by measuring the incorporation of a single pulse of BrdU.

Figure 3B depicts results that are representative of three independent experiments. Prior to induction, the cell density in each culture was similar for the parental S2-6 cells and the *Slfn1* transfected clone, 18-9. Approximately 30% of the cells were in S phase, and 9% were in G_2/M. Over the course of the experiment, the percentage of control S2-6 cells in S and G_2/M remained relatively constant until the cells reached confluence at 72 hr. Once confluent, these cells became quiescent and the percentage in S phase dropped. Conversely, induction of *Slfn1* in 18-9 cells reduced the percentage in S phase within 24 hr, and there was no further increase in the number of cells recovered after this time point. Interestingly, the percentage of 18-9 cells in S phase increased at 72 hr, even though the density remained

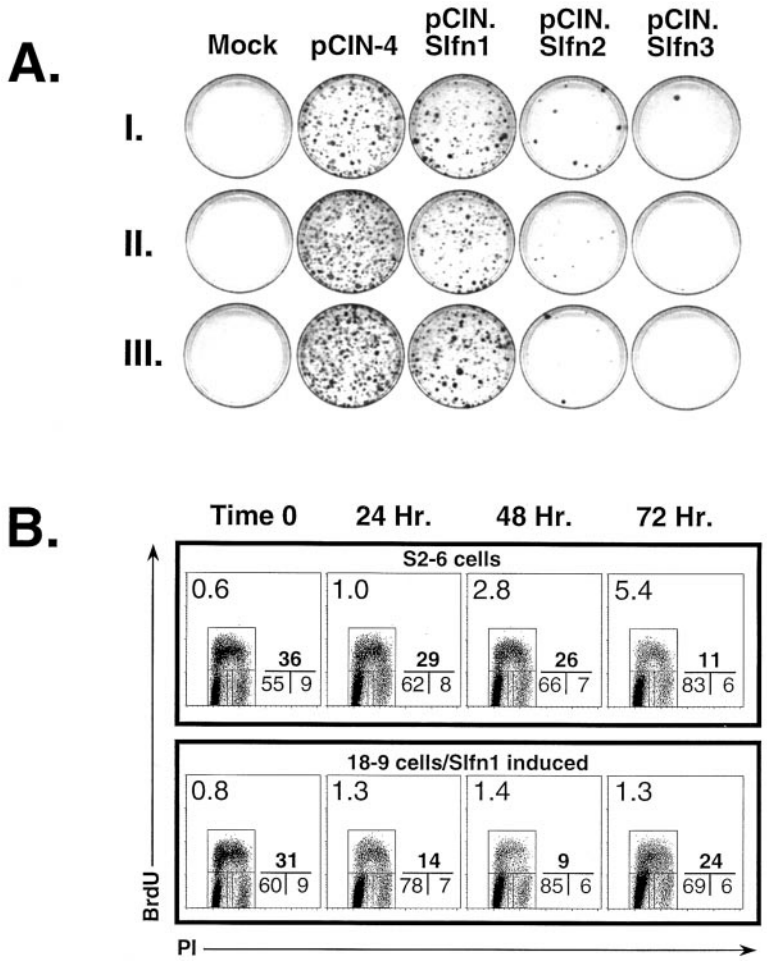

Figure 3. Effect of Ectopic *Slfn1* Expression on Cell Growth

(A) *Schlafen* cDNAs were cloned into pCIN-4, which encodes both the gene of interest and neomycin phosphotransferase in a single bicistronic message. NIH-3T6 fibroblasts were transfected with the indicated constructs and drug-resistant colonies were counterstained with hematoxylin.

(B) *Slfn1* was placed under control of the tetracycline operator and transfected into S2-6 fibroblasts that express a tetracycline responsive transactivator. *Slfn1* expression was induced by removing tetracycline for the indicated time intervals. The effect on the cell cycle of *Slfn1*-expressing cells (18-9) relative to parental cells (S2-6) was assessed by simultaneously measuring the incorporation of BrdU and propidium iodide. The number of cells recovered is indicated in the upper left-hand corner. In the lower right-hand corner, the percentage of cells in G_0/G_1 (lower left region), S (upper region), and G2/M (lower right region) are indicated.

unchanged. This is consistent with our observation of a gradual increase in 18-9 growth following 4 days of *Slfn1* expression (D.A.S. and S. Gray, data not shown). Collectively, these data demonstrate that *Slfn1* inhibits the G1 to S phase transition of the cell cycle. We detected no change in cell viability at any point during *Slfn1* expression, as assessed by either trypan blue uptake or the quantitation of subdiploid amounts of DNA. This appears to exclude apoptosis as the method by which fibroblast growth is disrupted. We have been unable to carry out similar experiments with *Slfn2* or *Slfn3* because we cannot obtain stable S2-6 transfectants. The basal expression of *Slfn2* or *3* in this system may be sufficient to disrupt cell growth.

Ectopic Early Expression of *Slfn1* Disrupts Thymic Development

Based on the observations that *Slfn1* causes a cell cycle arrest, we wanted to determine whether SLFN participates in regulating thymocyte expansion or maintaining mature thymocytes in a quiescent state. To accomplish this, we produced transgenic mice using a *Slfn1* cDNA under the transcriptional control of the human CD2 promoter/enhancer/locus control region. This combination of regulatory elements drives dose-dependent transgene expression in all thymocyte lineages including the $CD4^-8^-$ double negatives (DNs), and expression persists in mature peripheral T cells. Of 39 live births, two founder lines were identified (Slfn14 and Slfn17). Only the Slfn14 line (subsequently referred to as CD2.Slfn1) expressed the transgene. The level to which the transgene is expressed is slightly less than that detected for the endogenous message in normal peripheral lymphocytes (Figure 4A). However, in the thymus, expression of the transgene predominates, and presumably reflects the loss of the SP cells (see below), which are the primary source of endogenous *Slfn1* message (Figure 2C). We emphasize that *Slfn1* is not overexpressed in CD2.Slfn1 mice. Rather, we have altered the kinetics of its expression such that DP thymocytes express levels normally found in SP thymocytes.

The FACS profiles depicted in Figure 4B are representative of several independent experiments, while the number of cells in each subset were calculated from the collective data of 11 littermate, 13 CD2.Slfn1$^{+/-}$, and 7 CD2.Slfn1$^{+/+}$ mice. All mice ranged in age from 40–50 days, and identical quadrants were set in each experiment. As shown, early expression of *Slfn1* caused a severe reduction in overall thymus size, such that the thymi of CD2.Slfn1$^{+/-}$ mice contain only 10% to 30% of the thymocytes found in littermate control (LMC) mice. If expression was increased 2-fold by generating mice

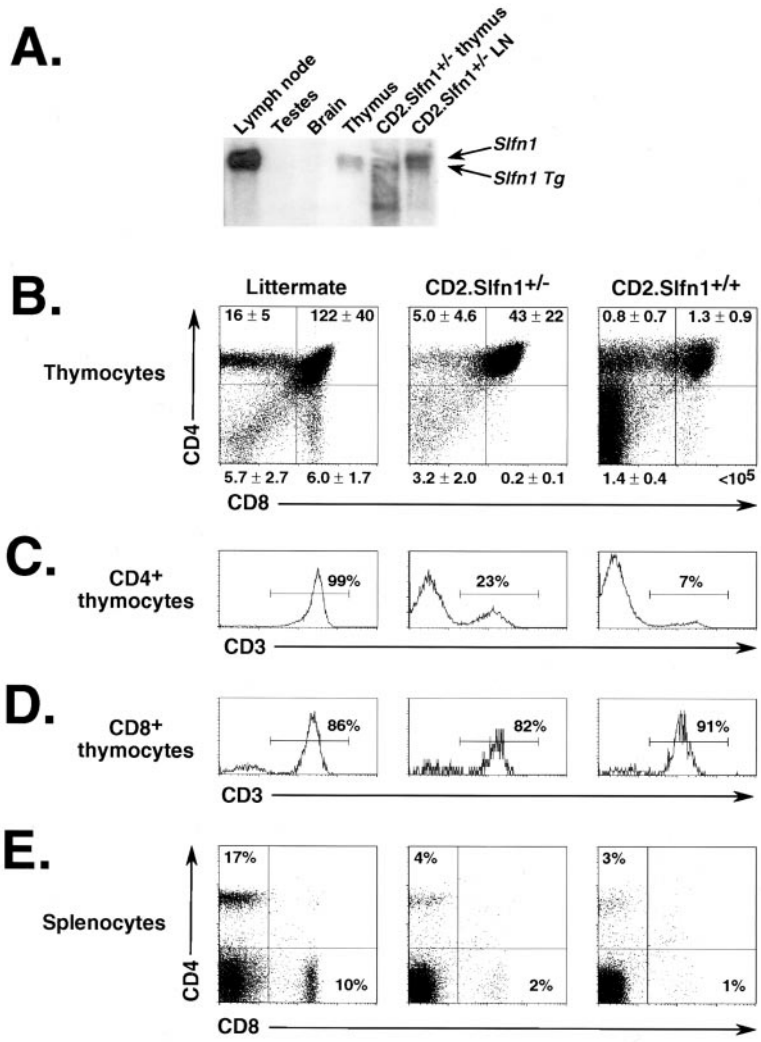

Figure 4. Effect of Ectopic *Slfn1* Expression on Thymocyte Development and Cellularity

(A) Steady-state message levels in the thymi and lymph nodes of CD2.Slfn1$^{+/-}$ transgenic mice were measured by probing a Northern blot with the *Slfn1* cDNA. RNA from select tissues of normal mice are included for comparison. The transgene encodes a message that is slightly smaller than its endogenous counterpart.

(B) The expression of CD4 and CD8 was measured on thymocytes from the indicated mice and the number of cells ($\times 10^{-6}$) representing each subset is shown.

(C and D) Electronic gates were set on the CD4 or CD8 subsets, respectively, and the expression of CD3 was measured.

(E) Representative data of CD4 and CD8 expression on splenocytes prepared from the indicated mice.

homozygous for the transgene, the thymi contained only 1%–3% of the cells found in LMC mice.

While ectopic expression of *Slfn1* reduced thymic cellularity as anticipated, there was also a severe alteration in development. This was readily evident from the thymus subpopulations (Figure 4B), and we found it to be exaggerated in the peripheral lymphoid organs (Figure 4E). Homozygous transgenic mice exhibited a profound loss of SP thymocytes and T cells. Although a large population of CD4 SP cells was found within the thymus of the transgenic mice, most of these cells appear to be immature since they expressed little or no CD3 (Figure 4C) but high levels of the heat stable antigen typically found on immature thymocytes (data not shown). This is in direct contrast to the small number of CD8 cells that are relatively normal in their expression of CD3 (Figure 4D). Interestingly, as the transgene dosage increases, there is a coordinate appearance of a population of CD4^{lo} cells that may be the precursor to the DN subset (Wu et al., 1991b). These cells may accumulate as a consequence of an early developmental block in thymocyte expansion, particularly since a similar population is observed in RAG and TCRβ chain–deficient mice, both of which contain thymocytes that are arrested early in development (Mombaerts et al., 1992a, 1992b). We know of no other transgene that evokes such a dramatic alteration in T cell development. Considering that the protein sequence is unaltered, the expression level is physiological, and the gene is naturally expressed (later) in the same lineage, this exquisite disruption of development is remarkable.

CD2.Slfn1 Disrupts Thymocyte Division

The reduction in thymic cellularity, particularly in CD2.Slfn1$^{+/+}$ mice, is consistent with a disruption in thymocyte progression through cell division. This was assessed by measuring the incorporation of a single in vivo pulse of the thymidine analog BrdU into DN thymocytes from wild-type or CD2.Slfn1$^{+/+}$ mice. Thymocytes were stained with antibodies to CD4, CD8, and BrdU (Figure 5A), and BrdU incorporation was measured by setting an electronic gate on the DN subset. Twenty percent of DN thymocytes from wild-type mice were positive for BrdU incorporation (Figure 5B), and this corresponds closely with published data (Penit et al., 1995).

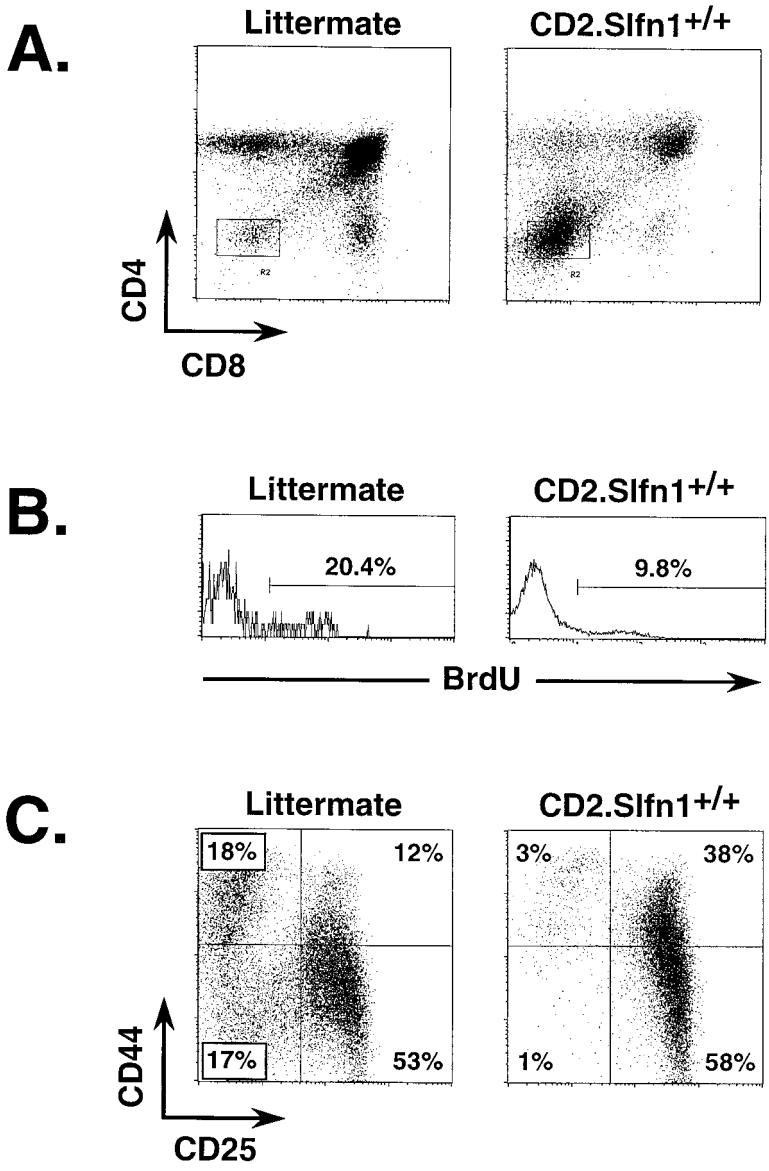

Figure 5. Effect of Ectopic *Slfn1* Expression on Thymocyte Proliferation

(A) Mice were administered a single dose of the thymidine analog BrdU, and 24 hr later thymocytes were fixed and triple-stained with antibodies specific for BrdU, CD4, and CD8. The expression of CD4 and CD8 is shown for the indicated mice.

(B) Electronic gates were set on the DN subsets shown in (A), and the percentage of BrdU positive cells was measured.

(C) Thymocytes were treated with anti-CD3, -CD4, and -CD8 plus complement and the resulting cells stained with antibodies specific for CD4, CD25, and CD44. The percentage of $CD4^-$ cells expressing the indicated markers is shown in each quadrant.

The ectopic expression of *Slfn1* inhibited the proliferation of the DN cells, as only 10% of these cells incorporated BrdU. Note that the fixation of the cells during staining caused a slight alteration in the bivariate profile (Figure 5A), making it impossible to distinguish the DN and $CD4^{lo}$ subset in the transgenic mice. However, since the $CD4^{lo}$ subset was shown to have an activated phenotype (Wu et al., 1991a), it is likely that these cells are cycling, and they would therefore be expected to increase the percentage of $BrdU^+$ cells in the DN subset. Thus, the overall inhibition of DN division may be greater than shown.

Immature DN thymocytes are developmentally heterogeneous, and these cells can be further fractionated based on the expression of CD25 and CD44. These markers alone define four distinct subsets within the DN subset (Figure 5C). The most immature cells express CD44 only, and these cells divide in response to intrathymic IL-7 (Peschon et al., 1994). As the cells mature, they appear to upregulate CD25 and then downregulate CD44. In the $CD25^+44^-$ subset, TCRβ chain rearrangement takes place, and a productive rearrangement promotes entry into the cell cycle (Falk et al., 1996; Hoffman et al., 1996). The cells subsequently downregulate CD25 to become $CD44^-25^-$. A similar analysis performed with CD2.$Slfn1^{+/+}$ mice revealed that the vast majority (58%) of $CD3^-4^-8^-$ thymocytes are arrested at the $CD25^+44^-$ stage of development (Figure 5C). This is the same stage at which RAG-deficient thymocyte development is blocked and is consistent with the idea that ectopic *Slfn1* expression interferes with the proliferative expansion of $CD25^-44^-$ thymocytes. Interestingly, there was also a reduction in the percentage of $CD25^-44^+$ cells. It may be that *Slfn1* expression also blocked the IL-7-driven proliferation of these cells. Alternatively, excess $CD25^+$ cells may have inhibited the expansion of the $CD25^-44^+$ cells through an unspecified feedback mechanism.

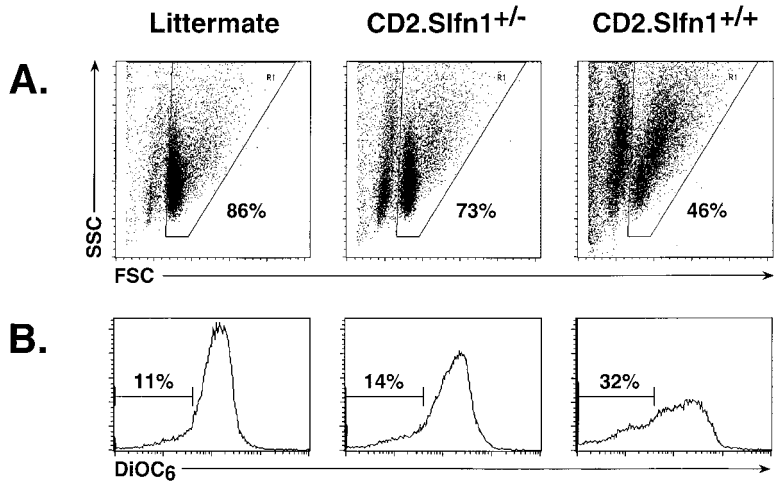

Figure 6. Effect of Ectopic *Slfn1* Expression on Thymocyte Viability

(A) Cell viability was measured by flow cytometry. Dead cells can be distinguished based on their decrease in cell size (forward light scatter [FSC]) and increase in granularity (90° light scatter [SSC]). The percentage of live cells is shown.

(B) Thymocytes were stained with $DiOC_6$, which measures alterations in the mitochondrial proton gradient. A decrease in $DiOC_6$ staining in the gated live cell population is indicative of cells undergoing early stages of apoptosis.

CD2.Slfn1 Thymocytes Are Apoptotic

The process of T cell development in the thymus is known to involve a high rate of cell death (Weissman et al., 1975; Jenkinson et al., 1989; Zacharchuk et al., 1991), although there is also a coordinate capacity for the engulfment of apoptotic thymocytes (Surh and Sprent, 1994). Free, apoptotic thymocytes are thus rare in freshly explanted thymocytes. However, using forward and side scatter parameters to measure cell size and granularity, we readily noticed an unusually large percentage of dead cells in thymi isolated from CD2.Slfn1 transgenic mice (Figure 6A). While 86% of thymocytes from LMC mice were viable, more than half the thymocytes were dead in CD2.Slfn1$^{+/+}$ mice. This was confirmed by measuring the extent of DNA fragmentation in the explanted thymocyte suspensions by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) (data not shown).

The morphological changes associated with apoptosis occur relatively late in the death pathway, whereas earlier stages of apoptosis can be detected by measuring changes in the proton gradient maintained across the inner mitochondrial membrane. As cells undergo apoptosis, the membrane potential drops, and this can be measured as a reduction in the intensity of $DiOC_6$ staining (Zamzami et al., 1995). As shown in the experiment depicted in Figure 6B, 32% of the viable cells from CD2.Slfn1$^{+/+}$ mice were $DiOC_6$ low as compared to 11% in thymocytes from a LMC mouse. Thus, using three independent measures, we found a significant increase in the number of apoptotic thymocytes isolated from *Slfn1* transgenic mice. In each case, the phenotype of CD2.Slfn1$^{+/+}$ mice was an exaggeration of that seen in CD2.Slfn1$^{+/-}$ heterozygotes. We conclude that the observed reduction in thymic cellularity appears to be a consequence not only of reduced cell division but of increased cell death.

***Slfn*1-Deficient Mice**

In order to determine whether *Slfn1* expression is required for normal regulation of thymocyte expansion, we produced mice with a homozygous deficiency in the *Slfn*1 gene (Figure 7). In embarking on this line of experimentation, our concern was that there are multiple members of the *Slfn* family, and at least *Slfn1-3* are potentially capable of inhibiting cell growth. However, if these members were not functionally redundant, we hypothesized that the loss of *Slfn1* would result in hyperproliferative disorders and perhaps even tumor formation.

The coding region for *Slfn1* is located on a single exon (Figure 7A) and it was targeted for deletion by a complete replacement with tk-neo. A total of four ES cell clones hemizygous for *Slfn1* were generated (Figure 7B), of which two led to germline transmission. Using rtPCR, we confirmed that there was no remaining *Slfn1* message in mice with a homozygous deficiency (Figure 7C). Mice were analyzed for changes in thymus cellularity or alterations in the expression of a variety of cell surface markers including CD4 and CD8 on both thymocytes (Figure 7D) and peripheral lymphocytes (Figure 7E). No phenotypic differences between *Slfn1*-deficient mice and their LMC counterparts have been noted thus far, and we conclude that *Slfn1* is not required for these aspects of lymphoid physiology. We suspect that one of the other family members may indeed be compensating for the loss of *Slfn1*. In particular, *Slfn2* is similar to *Slfn1* in both size and expression. We are currently attempting to address this issue by producing mice deficient for multiple *Slfn* family members.

Discussion

We have identified a novel gene family that consists, in mice, of at least seven highly related members. These genes encode proteins that fall into two distinct groups based on size; a short form encoded by *Slfn1* and *2* and a long form encoded by *Slfn3* through *7*. The prototype member of this family, *Slfn1*, was isolated in a screen designed to identify genes that are transcriptionally upregulated during the process of positive selection. While we have demonstrated a large increase in the expression of this gene during thymocyte development, we also found that there is a substantial difference in the expression of at least two other family members

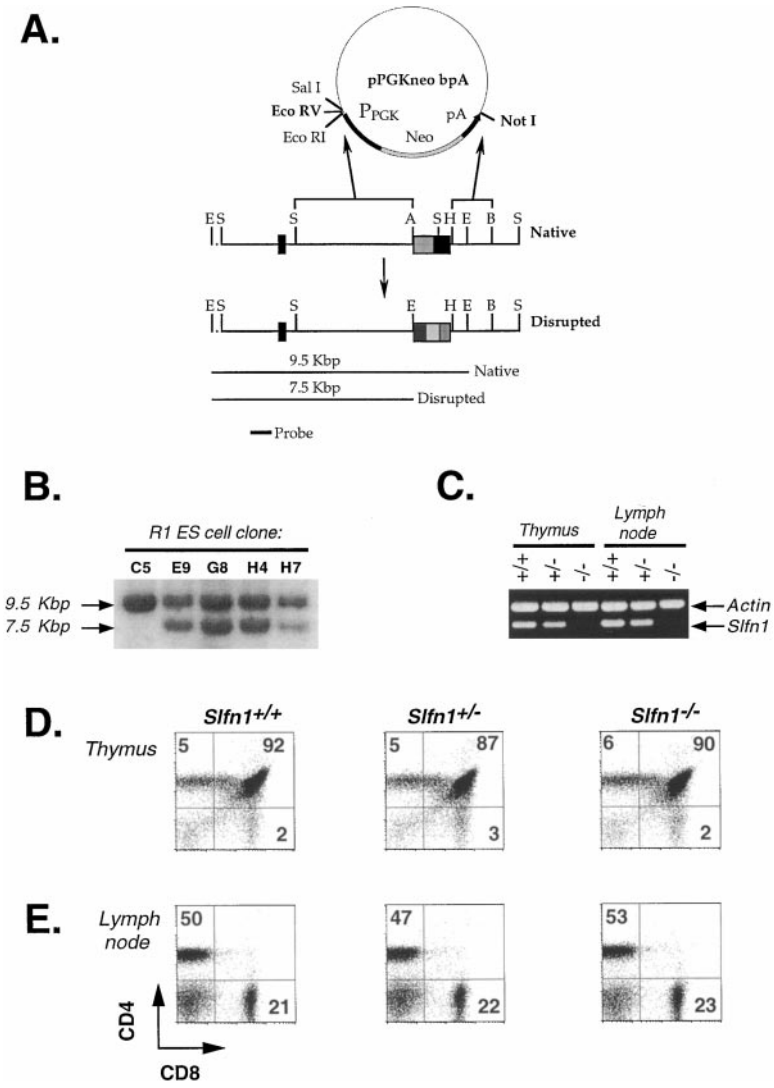

Figure 7. Analysis of *Slfn1*-Deficient Mice

(A) The second exon of *Slfn1*, which contains the putative ORF in its entirety (shaded box), was replaced with the neomycin cassette. E, EcoRI; S, SacI; A, AvaI; H, HindIII; B, BamHI.

(B) Four distinct *Slfn1* hemizygous ES cell clones were isolated, two of which (H7 and E9) resulted in germline transmission of the disrupted locus. (C) Total RNA was isolated from the thymi and lymph nodes of the H7 line of mice and subjected to RT-PCR using primers that span the 4.2 Kbp intron separating exons 1 and 2. Actin primers were included in each reaction to monitor amplification. Single cell suspensions were prepared from the thymus (D) and pooled lymph nodes (E) of H7 line mice. CD4 and CD8 expression was analyzed by flow cytometry and the percentage of cells in each quadrant is indicated.

during maturation. Comparing the DP and SP stages of development, both *Slfn1* and *2* are upregulated, whereas *Slfn 4* is downregulated. The precise point of regulation is not yet known but preliminary evidence indicates that it depends upon positive selection. For instance, there is a failure of *Slfn1* upregulation in two types of mice deficient in positive selection: MHC-deficient mice (D.A.S., J. Kaye, and S.M.H., unpublished data) and TCR transgenic mice with a nonselecting MHC haplotype (AND.4R TCR transgenic mice). We propose that changes in the expression of *Slfn1*, *2*, and *4* occur as a part of the program of mature T cell differentiation and may contribute to the phenotypic differences noted for each thymic subset.

Although early ectopic expression of *Slfn1* has a profound effect on thymocyte development, the most prominent attribute of this gene family is their effect on cell growth and progression through the cell cycle. Using a repressible promoter system to drive the expression of *Slfn1*, we found that the induction of *Slfn1* causes a cell cycle arrest such that cells fail to initiate DNA synthesis and are consequently arrested at the G_0/G_1 stage of the cell cycle. Furthermore, this growth inhibition can be reversed by terminating gene expression (data not shown). Hence, the family has been named *Schlafen*, which is the German word meaning "to sleep." The effects of *Slfn1* on cell growth in vitro are the weakest of the group as measured by colony counts in stable transfections, and, as mentioned above, thus far we have been unable to generate inducible versions of *Slfn2* or *3* using the tetracycline-repressible promoter.

The primary amino acid sequence for each of the four family members provides no clue to their potential function. We have also used The Jackson Laboratories C57BL/6 × *M. spretus* interspecific backcross panel (Rowe et al., 1994) to map the chromosomal location of both *Slfn1* and *2*. Both of these genes map to a position on mouse chromosome 11 that lies in the vicinity of the CC chemokine family (D.A.S., unpublished data); however, they bear no similarity to this family other than having two adjacent cysteines. Thus, none of the data collected to date have provided a clear picture on the mechanism by which the SLFN molecules exert their effect. Despite this, the preponderance of data thus far

suggests that the SLFN molecules almost certainly participate in the regulation of the cell cycle. These data include: (1) the cytostatic effects seen in fibroblasts and AKR1 cells; (2) a reduction in the expression of *Slfn1* and *2* following induction of T cell proliferation; (3) the loss of *Slfn1* expression, and to a lesser extent *Slfn2* expression, in long-term T cell tumors and clones (data not shown); and (4) the reduction in thymic cellularity observed in CD2.Slfn1 transgenic mice.

Although we have demonstrated that the reduction in thymic cellularity can be attributed at least in part to a decreased rate of cell division, we also found an unusually high amount of cell death in freshly explanted thymi. Because of the block in cell growth imposed by *Slfn1*, this death may result from inductive stimuli received through the TCR that are subsequently unable to initiate progression through the cell cycle. This is reminiscent of the apoptosis observed when *c-myc* is induced in growth-arrested cells (Evan et al., 1994; Dou et al., 1995; Morse et al., 1997; Murata et al., 1997). In fact, *c-myc* expression is differentially regulated during thymocyte maturation; the highest levels are found in the TN thymocytes, and expression decreases in DP cells expressing low levels of CD3 prior to being upregulated again in the mature SP cells (Broussard-Diehl et al., 1996). Since *c-myc* is expressed at very high levels at the same developmental stage in which proliferation is blocked in the transgenic mice, this is likely to account for the massive death seen in these thymi.

As of now, we cannot put forth a mechanistic hypothesis to explain the function of the *Slfn* gene products. They clearly must be carefully regulated since relatively small changes in expression can completely disregulate cell growth and cell death. SLFN must act on the fundamental machinery involved with cell cycle control since even the lymphocyte-specific members can arrest growth of fibroblasts. Beyond this, we need to know how SLFN perturbs the cell cycle, and the identity of the molecules with which it interacts. Although the primary sequences of the hypothetical proteins have not revealed any mechanistic secrets, perhaps the solution of the tertiary protein structure or the identification of interacting proteins would suggest a function. Our conclusion is that this novel family of proteins must participate in a heretofore unknown regulatory mechanism guiding both cell growth and T cell development.

Experimental Procedures

Isolation and Sequencing of *Schlafen* cDNAs

PolyA$^+$ RNA from AND.B6 and AND.4R thymocytes was isolated using a QuickPrep Micro mRNA purification kit (Pharmacia). Complementary DNA libraries were prepared using a Superscript plasmid cDNA library kit (GIBCO-BRL) according to the manufacturer's recommended protocol. Inserts excised from the plasmid backbone were used for subtractive hybridization using a previously described protocol (Hara et al., 1994). Subtracted material was subcloned into pBK-CMV (Stratagene), and a single cDNA clone encoding *Slfn1* (GenBank AF099972) was recovered. Subclones of the *Slfn1* cDNA were sequenced on both strands using the Sanger dideoxy chain termination method. The orientation and placement of each subclone was determined by restriction mapping or by sequencing additional overlapping clones.

To isolate *Slfn2* and *3*, a C57BL/6 thymocyte cDNA library was prepared in the λ vector UNI-ZAP (Stratagene). The library was hybridized at high stringency with an 800 bp EcoRI/NotI fragment from the I.M.A.G.E. consortium clone 332469 (GenBank W08491), and 12 individual clones were isolated. Subclones in pBluescript SK(-) were excised with helper phage and sequenced from both ends. Eleven of the twelve clones shared identity with the EST clone 332469 (*Slfn2*), while only one was different (*Slfn3*). The longest *Slfn2* cDNA (GenBank AF099973) and *Slfn3* (GenBank AF099974) were sequenced completely on both strands.

To derive the amino acid sequence of *Slfn4*, the I.M.A.G.E. consortium clone 638739 was sequenced on both strands (GenBank AF099975). The oligonucleotides Slfn3B.5 and Slfn4/430 were then used in rtPCR to amplify a partial clone from splenic polyA$^+$ RNA (GenBank AF099976). This clone was sequenced on both strands and compared to a genomic clone to account for errors introduced by either reverse-transcriptase or *Taq* polymerase. Based on these sequences, two new oligonucleotides were synthesized (Slfn4/385 and Slfn4/-52) that were used to sequence a genomic clone encoding the presumptive translation initiation site and an upstream stop codon (GenBank AF099977).

To compare the deduced amino acid sequence of each gene, the sequences were first aligned using Clustal W version 1.5 (Thompson et al., 1994) and then imported into SeqVu version 1.0 (The Garvan Institute of Medical Research) for graphical presentation.

Purification and Activation of Peripheral T Cells

Lymphocytes were pooled from the lymph nodes of 4- to 8-week-old adult mice and resuspended in RPMI complete (RPMI-1640, 10% FCS, 1 mM sodium pyruvate (Irvine Scientific), 1× nonessential amino acids (Irvine Scientific), 2 mM L-glutamine, 5×10^{-5} 2-mercaptoethanol, and antibiotics). The cells were adsorbed to plastic for 1 hr at 37°C to remove macrophages and dendritic cells, and B cells were removed using sheep anti-mouse IgG magnetic beads (Dynal). Six centimeter dishes were coated with 10 μg/ml goat anti-hamster Ig for 1 hr at 37°C in carbonate buffer. Plates were washed, and purified anti-CD3 (145.2C11) was added at a concentration of 100 ng/ml in growth media and allowed to incubate for 1 hr at 37°C. Plates were washed again and 3×10^6 cells were added in the presence or absence of a 1:20,000 dilution of anti-CD28 ascites (Gross et al., 1992) and cultured for the indicated time period at 37°C.

Purification of Thymocyte Subsets

Thymocytes from adult B10.A female mice were treated for 45 min with Low-tox rabbit complement (Cedarlane Laboratories) and antibodies specific for CD4 (RL172) and/or CD8 (3.168.36). Treated cells were then stained on ice for 1 hr (see below) and the appropriate subsets electronically sorted using a Becton-Dickinson FACStar. Alternatively, CD3$^-$4$^-$8$^-$ cells were purified by treating with complement and the same antibodies described above supplemented with the anti-CD3 antibody C363-29B.

Preparation and Analysis of RNA

Total RNA was prepared using either Trizol reagent (GIBCO-BRL) or RNeasy spin columns (Qiagen) following the manufacturer's protocol. For Northern blot analysis, 30 μg of total RNA was resolved through a 1% formaldehyde gel, transferred to NitroPure nitrocellulose (Micron Separations Incorporated), and hybridized to a radiolabeled *Slfn1* cDNA probe. For rtPCR analysis, total RNA was treated with Rnase-free Dnase and converted to cDNA with AMV reverse transcriptase. Two hundred nanograms (or 2×10^5 cell equivalents in the case of the thymocyte subsets) of the resulting samples were subjected to 30 cycles of the PCR in a 25 μL buffered solution containing 1.5 mM Mg^{++} and either 10 pmoles (SlfnORF5, Slfn2B.5, Slfn3D.5, Slfn4.5, β-Actin3, and β-Actin5) or 30 pmoles (Slfn4/B) primer. For analysis of *Slfn1*-deficient mice, 10 pmoles each of 18.RTP3, 18.RTP5, β-Actin3, and β-Actin5 primers were used. The individual cycles were 20 sec at 95°C, 40 sec at 57°C, and 60 sec at 72°C. One half of the amplified material was resolved through an agarose gel containing EtBr and the image captured electronically using IPLab Gel H software (Signal Analytics). In the case of activated T cells, a profile plot representing band intensity was generated for each PCR product series (for example, *Slfn1* at each time point) using IPLab Gel H software. The area (A) of each peak was

then calculated. For each time point (for example, undiluted cDNA at time 0), the area taken up by actin was normalized relative to the area of the most intense actin band in the series. The resulting "normalization coefficient" was used to normalize the areas calculated for the other *Slfn* products in that cDNA sample.

Oligonucleotides

All oligonucleotides used in this study were synthesized by the GENSET Corporation and are listed beginning at the 5′ end. SlfnORF5 (ATGAACATCACCGATGAAGGG); Slfn2B.5 (CTCAGAAAACAGGAGAATGC); Slfn3B.5 (CCGGTATCATGCACCTTCC); Slfn3D.5 (ATCAACTCAATCTCAGATGAAG); Slfn4.5 (GCAGTTCCTCAAATCCAGAC); Slfn4/B (GAAGTGAGTGACAGGCAGC); Slfn4/430 (GCTAAGGGCTAAATGTCG); Slfn4/-52 (AGTTGGTTTTCCAGGTGG); Slfn4/385(TAGCAGCTCAGATGGTTG); β-Actin3 (CTCTTTGATGTCACGCACGAT); β-Actin5 (GTGGGCCGCTCTAGGCACCAA); 18.RTP5 (AACGTGCTCAGTAGAGCAGC); and 18.RTP3 (TTCCCAACAGAGACATCTGG).

Expression of *Schlafen* cDNAs

Complementary DNA clones were subcloned into pCIN4 to generate the constructs pCIN.Slfn1, pCIN.Slfn2, and pCIN.Slfn3. Lipofectamine (GIBCO-BRL) was used to transfect plasmids into NIH-3T6 cells that were then maintained for 12–14 days in RPMI complete (see above) containing 500 μg/mL active G418. Drug-resistant colonies were counterstained with Mayer's Hematoxylin and photographed. For inducible expression of *Slfn1*, the cDNA was subcloned into pTet-Splice (GIBCO-BRL) and cotransfected along with pBK-CMV into S2-6 cells expressing the tetracycline transactivator. Stable transformants were selected with 650 μg/ml active G418 in the presence of 0.5 μg/ml tetracycline, and one of ten individual clones (18–9) was selected for further study.

Production of Transgenic Mice

The *Slfn1* cDNA was cloned into pTex (Lake et al., 1990) and a fragment containing *Slfn1* under control of the human CD2 promoter and enhancer was purified over sucrose. C57BL/6 × BALB/c F2 embryos were microinjected with insert and the embryos were transplanted into pseudopregnant CD1 females. Two founders (Slfn14 and Slfn17) were produced from 39 mice, and SLFN14 has been maintained by backcrossing to C57BL/6 mice.

Production of *Slfn1*-Deficient Mice

The construct depicted in Figure 7 was linearized with SalI and transfected into the R1 ES cell line by electroporation. Transfected cells and resulting clones were grown on neomycin-resistant mouse embryonic fibroblasts that were inactivated with mitomycin c. *Slfn1*-deficient clones were identified by digesting genomic DNA with EcoRI and screening with a radiolabeled PvuII fragment from a region flanking the 5′ end of exon 1. Recombinant clones were microinjected into C57BL/6 blastocysts, and chimeric mice were backcrossed to C57BL/6 mice. DNA was isolated from tail snippets of agouti pups and screened as described above to identify mice hemizygous for the *Slfn1* disruption.

Flow Cytometry

One million thymocytes were washed in PBS containing 2% fetal bovine serum and 0.1% NaN_3 (omitted for sorting) and resuspended in the same containing saturating concentrations of one or more of the following antibodies: CD3-FITC (PharMingen); CD4-PE (Caltag); CD8-Tricolor (Caltag); CD25-FITC (PharMingen), and CD44-PE (PharMingen). Following a 30 min incubation at room temperature, the cells were washed once, and 50–100,000 events were collected on a FACScan using CellQuest software (Becton-Dickinson). Forty nanomolar $DiOC_6$ was added with saturating antibodies where indicated, and the incubation was carried out for 15 min at 37°C. In vivo BrdU incorporation was measured as described (Carayon and Bord, 1992). In brief, mice were injected IP with 1 mg BrdU and 1×10^6 thymocytes were stained as described above with CD4-PE and CD8-Tricolor. Cells were fixed in 1% paraformaldehyde, permeabilized in 70% EtOH, and treated with 50 Kunitz units DNase prior to the addition of 10 μL of FITC-conjugated anti-BrdU (Becton Dickinson). To monitor cell growth following conditional expression of *Slfn1*, 5×10^5 cells were plated into 10 cm tissue culture dishes and allowed to adhere overnight. Cells were then washed twice and fresh media lacking tetracycline was added. Thirty minutes prior to harvesting, the cultures were pulsed with 10 μM BrdU. After staining fixed cells with 10 μL FITC-conjugated anti-BrdU and 5 μg/ml propidium iodide (Dolbeare et al., 1983), they were analyzed by flow cytometry.

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The GenBank accession numbers for the sequences reported in this paper are AF099972, AF099973, AF099974, AFO99975, AFO99976, and AFO99977.