Role of Schlafen 2 (SLFN2) in the Generation of Interferon α-induced Growth Inhibitory Responses*

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The precise STAT-regulated gene targets that inhibit cell growth and generate the antitumor effects of Type I interferons (IFNs) remain unknown. We provide evidence that Type I IFNs regulate expression of Schlafens (SLFNs), a group of genes involved in the control of cell cycle progression and growth inhibitory responses. Using cells with targeted disruption of different STAT proteins and/or the p38 MAP kinase, we demonstrate that the IFN-dependent expression of distinct Schafen genes is differentially regulated by STAT complexes and the p38 MAP kinase pathway. We also provide evidence for a key functional role of a member of the SLFN family, SLFN2, in the induction of the growth-suppressive effects of IFNs. This is shown in studies demonstrating that knockdown of SLFN2 enhances hematopoietic progenitor colony formation and reverses the growth-suppressive effects of IFNα on normal hematopoietic progenitors. Importantly, NIH3T3 or L929 cells with stable knockdown of SLFN2 form more colonies in soft agar, implicating this protein in the regulation of anchorage-independent growth. Altogether, our data implicate SLFN2 as a negative regulator of the metastatic and growth potential of malignant cells and strongly suggest a role for the SLFN family of proteins in the generation of the antiproliferative effects of Type I IFNs.

Type I interferons (IFNs) are potent inhibitors of cell growth of both normal and malignant cells in vitro and in vivo and play critical roles in the immune surveillance against cancer (1–4). The potent antitumor properties of Type I IFNs have prompted extensive efforts over the years to understand the mechanisms by which these cytokines generate signals and induce biological responses. Key events elicited during engagement of the Type I IFN-receptor have been identified, and major signaling cascades that are activated in an IFN-dependent manner have been defined. The Jak-STAT pathway is the most important pathway in the regulation of IFN-inducible gene transcription and probably the best studied and characterized IFNα-regulated signaling pathway to date (reviewed in Refs. 2 and 5–7). Beyond the Jak-STAT pathway, other highly relevant cellular cascades in IFN signaling are MAP kinase pathways (8–13) that control auxiliary signals for optimal gene transcription and Akt/mTOR pathways that promote mRNA translation of IFN-stimulated genes (ISGs) (14–18). An emerging model for the production of Type I IFN-inducible gene products involves transcriptional regulation of ISGs by Jak-STAT pathways, immediately followed by mRNA translation of such transcripts in an mTOR/4EBP1-dependent manner (17, 18).

The identification and definition of Type I IFN receptor-generated signals that promote transcription and mRNA translation of target genes has provided critical information of how early signals at the receptor level ultimately translate to Type I IFN responses. A remaining challenge in the IFN signaling field is the identification of specific genes or groups of genes that specifically account for the induction of the diverse biological responses of IFNs. Various proteins that are involved in the generation of the antiviral effects of IFNs have been identified over the years (19). However, very little is known on ISG products that participate in the generation of IFN-dependent antiproliferative responses. In fact, the key IFN-inducible gene products that mediate growth inhibitory responses in different cell types remain largely unknown.

The Schlafen (SLFN) (from the German word schlafen or sleeping) family of proteins includes several members that have previously been shown to control cell cycle progression and growth arrest (20–26). These proteins contain a common N-terminal (AAA) domain that is involved in GTP/ATP binding (20, 22), whereas a subgroup of these proteins, the long SLFNs, have motifs found in members of Superfamily I of DNA/RNA helicases (21). There is evidence that Schlafen proteins promote growth inhibitory responses (20) and modulate cell cycle progression by inhibiting cyclin D1 (22). Although limited studies have been conducted on the roles of distinct Schlafen group members on the regulation of cellular functions, there is emerging evidence indicating a potentially important role for these proteins in the control of cell cycle progression. Regardless, very little is known on the potential involvement of SLFN genes and their products in the induction
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of antiproliferative responses induced by IFNs or other growth-suppressive cytokines.

In the present study we examined the induction of expression of various mouse SLFN family members during treatment of sensitive cells with IFNα. Our data demonstrate that SLFN1 and SLFN2 (group I), SLFN3 (group II), as well as SLFN5 and SLFN8 (group III) are all genes inducible by treatment of sensitive cells with mouse IFNα. Using defined knock-out cells for different STAT proteins and/or the p38 MAP kinase, we provide evidence for differential regulation of distinct SLFN members by different STAT complexes and the p38 MAP kinase. In other studies we provide evidence that knockdown of SLFN2 enhances murine hematopoietic progenitor colony formation and reverses the growth-suppressive effects of IFNα and IFNγ on normal hematopoiesis. In addition, our data show that NIH3T3 and L929 fibroblast cells with stable knockdown of SLFN2 form more colonies in soft agar compared with control cells, implicating this member of the SLFN family of proteins in the regulation of anchorage-independent growth. Altogether, our results indicate that SLFN2 acts as a negative regulator of the metastatic and growth potential of malignant cells, and it is an effector element in the generation of Type I IFN-induced antiproliferative responses.

MATERIALS AND METHODS

Cells Lines and Antibodies—NIH3T3 and L929 cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum and antibiotics. Immortalized mouse embryonic fibroblasts (MEFs) from p38α−/− knock-out mice (27) were kindly provided from Dr. Angel Nebreda (CNIO (Spanish National Cancer Center), Madrid, Spain). Immortalized STAT1 knock-out (28) and STAT3 knock-out (29) MEFs were generously provided by Dr. David Levy (New York University, New York, NY). In the figures, STAT3 WT refers to STAT3box−/− MEFs (29), whereas STAT3 KO MEFs refers to MEFs resulting from deletion of exons 16–21 of STAT3 by transfection of Cre recombinase (29). The different MEFs were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. A custom-made polyclonal antibody against the N-terminal region (amino acids 1–14) of mouse SLFN2 was produced and purified via New England Peptide LLC (Gardner, MA). Antibodies against Cyclin D1, Cyclin D3, CDK 4, CDK 6, p15 INK, and p27 KIP were obtained from Cell Signaling Technology (Danvers, MA). An antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Chemicon International (Temecula, CA). An antibody against Lamin A was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Lines—Mouse hematopoietic progenitor colony formation was assessed as previously described (12, 37). Colony formation assays were performed using Sca1+ cells isolated from mouse bone marrow stem cells according to the manufacturer’s instructions (MACS kit, Miltenyi Biotec Inc., CA). The cells were plated in methylcellulose media (Stemcell Technologies, Seattle, WA) in the presence or absence of 103 IU/ml IFNα, and colony formation was assessed after 7 days of culture. Anchorage-independent growth was assessed in soft agar assays in duplicate, carried out essentially as previously described (38). Briefly, the cells were suspended in 0.3% top agar over a bottom layer of 0.5% agar in 6-well plates. The solidified soft agar was overlaid with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics. The medium was changed every 4–5 days. The colonies were scored after 11 days (NIH3T3 cells) or 8 days (L929 cells) of culture.

mRNA Isolation and Real Time PCR Probes and Primers—Cells were treated with 5 × 103 IU/ml of IFNα for the indicated times and RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) and stored at −80 °C. The expression of mRNA was analyzed using Taqman Low Density Arrays (Applied Biosystems). The custom-made arrays were purchased from Applied Biosystems and the data were analyzed using the Cp method (Applied Biosystems).

siRNA Transfection and Generation of Stable SLFN2 Knockdown Cells—Transient knockdown of SLFN2 was performed using either SLFN2 ON-TARGETplus SMARTpool siRNA (SLFN2 siRNA1) and nontargeting control pool siRNA (Ctrl siRNA1) (Thermo Fisher Scientific, Waltham, MA) or a Silencer select SLFN2 siRNA pool (SLFN2 siRNA) and a Silencer select control nontargeting siRNA (Ctrl siRNA2) (Applied Biosystems, Foster City, CA). The siRNA transfection reagent TransIT-TKO was used according to the manufacturer’s instructions (Mirus Bio Corporation, Madison, WI). For the generation of stable SLFN2 knockdown NIH3T3 and L929 cells, a commercially available system from Clontech was used. Briefly, SLFN2 ON-TARGETplus SMARTpool siRNA and control scrambled sequences were used as templates in the Clontech shRNA sequence designer tool for Clontech pSIREN vectors. Plasmids were sequenced to verify the presence of siRNA encoding insert and then used for retroviral infection of NIH3T3 and L929 cells. Infected pSIREN-shRNA expressing cells were green fluorescent and were selected by flow cytometry.

Cell Proliferation Assays—Cell proliferation assays using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide method were performed as in our previous studies (35, 36).

Hematopoietic Progenitor Cell Assays and Soft Agar Assays—Mouse hematopoietic progenitor colony formation was assessed as previously described (12, 37). Colony formation assays were performed using Sca1+ cells isolated from mouse bone marrow stem cells according to the manufacturer’s instructions (MACS kit, Miltenyi Biotec Inc., CA). The cells were plated in methylcellulose media (Stemcell Technologies, Seattle, WA) in the presence or absence of 103 IU/ml IFNα, and colony formation was assessed after 7 days of culture. Anchorage-independent growth was assessed in soft agar assays in duplicate, carried out essentially as previously described (38). Briefly, the cells were suspended in 0.3% top agar over a bottom layer of 0.5% agar in 6-well plates. The solidified soft agar was overlaid with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics. The medium was changed every 4–5 days. The colonies were scored after 11 days (NIH3T3 cells) or 8 days (L929 cells) of culture.

mRNA Isolation and Real Time PCR Probes and Primers—Cells were treated with 5 × 103 IU/ml of IFNα for the indicated times. Isolation, purification of mRNA, and conversion into cDNA was performed using the respective kits and oligo(dT)s...
from Qiagen according to the manufacturer’s instructions. Validated, inventoried probes and primers for real time PCR and TaqMan PCR master mix were purchased from Applied Biosystems (Foster City, CA). The probes and primers were:

- **SLFN1**, Mm00488306_m1;
- **SLFN2**, Mm00488307_m1;
- **SLFN3**, Mm00488309_g1;
- **SLFN5**, Mm00806095_m1;
- **SLFN8**, Mm00824405_m1; and
- **ISG15**, Mm01705338_s1.

**GAPDH** (Mm99999915_g1) was used as an internal control.

**RESULTS**

In initial studies we determined whether treatment of cells with IFNα induces expression of different SLFN genes. NIH3T3 cells were treated with mouse IFNα for different times, and the induction of mRNA expression for key members of the SLFN gene family was determined. As shown in Fig. 1, mRNA expression for different SLFN genes was inducible at various degrees in response to IFNα treatment. The most pronounced induction was for **SLFN1** (Fig. 1A), followed by **SLFN5**, **SLFN2**, and **SLFN8** (Fig. 1, B, D, and E). **SLFN3** was induced clearly to a lesser degree than other SLFNs (~4-fold), but its induction was consistently seen (Fig. 1C).

To better understand the regulation of SLFN proteins during engagement of the Type I IFN receptor, we generated and used an anti-SLFN2 antibody to directly examine the expression of SLFN2 protein after IFNα treatment of cells. This antibody was custom-generated via a commercial vendor against a conserved...
FIGURE 2. Differential requirement for STAT1 in IFNα-inducible expression of distinct SLFN mRNAs. STAT1^+/+ and STAT1^-/- MEFs were treated with IFNα for the indicated times. Total RNA was isolated and the expression of SLFN1 (A), SLFN2 (B), SLFN3 (C), SLFN5 (D), and SLFN8 (E) mRNAs was determined by real time RT-PCR, after normalization for GAPDH expression. The data are expressed as fold increases over control untreated samples and represent the means ± S.E. of two independent experiments for SLFN3 and SLFN5, three for SLFN8, four for SLFN1, and seven for SLFN2.
region in the N terminus of the protein and detects a single band at \( \sim 44 \) kDa, which is consistent with the predicted molecular mass of SLFN2. As shown in Fig. 1F, base-line expression of SLFN2 in NIH3T3 cells was clearly detectable, but treatment of cells with IFN\( \alpha \) resulted in up-regulation of the expression of the protein (Fig. 1F). We also examined the sub cellular localization of the protein. In a previous study, it was shown that overexpressed FLAG-tagged SLFN2 in HEK-293T cells is
Role of p38 MAPK in the regulation of expression of SLFN genes. p38α+/+ and p38α−/− MEFs were treated with IFNα for the indicated times. Total RNA was isolated and the expression of SLFN1 (A), SLFN2 (B), SLFN3 (C), SLFN5 (D), and SLFN8 (E) mRNAs was determined by real time RT-PCR, after normalization for GAPDH expression. The data are expressed as fold increases over control untreated samples and represent the means ± S.E. of three independent experiments for SLFN2, four for SLFN1 and SLFN3, and five for SLFN5 and SLFN8.
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exclusively expressed in the cytoplasm (25). However, FLAG tagging could theoretically interfere with the structural properties and localization of the protein, and the potential translocation of endogenous SLFN2 in response to cytokine treatment has not been known. In studies in which the localization of the endogenous protein was directly determined using the newly generated anti-SLFN2 antibody, we found that endogenous SLFN2 is exclusively expressed in the cytoplasm, and IFNα treatment does not induce its translocation to the nucleus (Fig. 1G).

IFNα binding to the type I IFN receptor results in activation of STAT1, STAT2, and STAT3 transcription factors, which form various homo- and/or heterodimers that can bind specific sequences in the promoters of IFN inducible genes (2–7). In addition, IFNα-mediated gene transcription is regulated by auxiliary pathways, such as the p38 MAPK pathway (7–13). To define the roles of distinct STAT proteins and the p38 MAPK in SLFN gene expression, experiments were performed using cells with targeted disruption of STAT1, STAT3, or p38α genes. In initial studies, STAT1−/− MEFs and STAT1+/+ parental MEFs were treated with mouse IFNα, and mRNA expression for SLFN1, SLFN2, SLFN3, SLFN5, and SLFN8 was determined. IFNα-dependent expression of all SLFN genes was decreased in STAT1 knock-out MEFs compared with parental MEFs, and the effect ranged from a partial impairment (SLFN3) to completely defective transcription (SLFN1, 2, 5, and 8) (Fig. 2). Similarly, IFNα-inducible SLFN expression was examined in STAT3 knock-out MEF cells. The induction of expression of SLFN1, SLFN2, SLFN3, and SLFN8 genes was decreased in STAT3 knock-out cells although not abrogated (Fig. 3, A–C). The expression of SLFN5 was completely STAT3-independent, and in fact, SLFN5 expression was enhanced in STAT3 knock-out cells (Fig. 3D).

p38 MAPK-activated signaling cascades play important roles in Type I IFN-dependent transcriptional regulation, acting as auxiliaries to STAT pathways, and their function is essential for full transcriptional activation of ISGs (reviewed in Refs. 7 and 39). To determine the role of p38α MAPK-mediated signals in SLFN gene expression, we used MEF cells with targeted disruption of the p38α gene (27) in which we have previously shown that IFNα-inducible transcription via ISRE or GAS elements is defective (33). IFNα-dependent mRNA expression for SLFN1, SLFN2, and, to a lesser degree, SLFN3 was suppressed in the absence of p38α MAPK (Fig. 4, A–C). On the other hand, the group III schlafen genes, SLFN5 and SLFN8, were induced by IFNα in a p38α MAPK-independent manner (Fig. 4, D and E), suggesting that p38 activity is essential for IFN-dependent expression of group I and II but not group III Schlafen genes.
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**A**

![Graph showing the expression of SLFN2 and SLFN1 mRNAs in pSIREN Zsgreen-SLFN2 siRNA and pSIREN Zsgreen control-siRNA NIH3T3 cells](image)

**B**

![Diagram showing the generation of IFN responses](image)

**C**

![Image showing the effects of IFN on NIH3T3 cells](image)

**FIGURE 6. Stable knockdown of SLFN2 enhances cell proliferation and impairs IFN-α-dependent growth inhibitory responses but has no effects on the generation of antiviral responses.**

**A**, expression of SLFN2 or SLFN1 mRNAs in pSIREN Zsgreen-SLFN2 siRNA and pSIREN Zsgreen control-siRNA NIH3T3 cells was determined by real time RT-PCR using specific primers and GAPDH as an internal control. The data are presented as percentages of expression in pSIREN Zsgreen control-siRNA cells and represent the means ± S.E. of three experiments. **B**, total cell lysates from pSIREN Zsgreen SLFN2-siRNA or pSIREN Zsgreen control-siRNA NIH3T3 cells were resolved by SDS-PAGE and immunoblotted sequentially with anti-SLFN2 or anti-GAPDH antibodies. The migration of the different STAT complexes is indicated by arrows. **C**, wild-type NIH3T3 cells, pSIREN Zsgreen SLFN2 siRNA NIH3T3 cells, or pSIREN Zsgreen control-siRNA NIH3T3 cells were treated with IFNα for the indicated times. Expression of **Isg15** mRNA was determined by real time RT-PCR using G3PDH as an internal control. The data are expressed as the means ± S.E. of three experiments.

It is well known that Type I and II IFNs are potent regulators of normal and leukemic hematopoiesis and inhibit the growth of primitive hematopoietic precursors *in vitro* and *in vivo* (4, 39, 40). It has been also established that activation of the p38 MAP kinase pathway is required for the generation of the myelosuppressive effects of IFNs on both normal and leukemic progenitors (12, 13). Because SLFN2 is induced by IFNs and its expression is regulated via both STAT and p38 MAPK pathways, we examined whether this protein plays a role in the generation of the myelosuppressive effects of IFNs. In initial experiments, two different specific siRNAs (Fig. 5A) were used to knock down SLFN2 expression in murine bone marrow-derived Sca1+ stem cells. Primitive progenitor colony formation was subsequently assessed in clonogenic assays in methylcellulose. Knockdown of SLFN2 in normal hematopoietic progenitors resulted in increased hematopoietic colony formation (Fig. 5, B–D), suggesting that this protein plays a critical role in the control of normal hematopoietic progenitor cell growth. Also, as expected, treatment of cells with IFNα (Fig. 5, C and D) or IFNγ (data not shown) resulted in suppression of hematopoietic progenitor colony formation compared with untreated cells, although the suppressive effects of IFNα were much less noticeable in cells in which SLFN2 was knocked down (Fig. 5, C and D). Thus, SLFN2 participates in the control of normal hematopoiesis and the generation of the myelosuppressive effects of IFNs, suggesting that this protein may be an effector in the regulation of p38-mediated hematopoietic suppression.

To further analyze the functional relevance of SLFN2 in cell growth regulation and its role in the generation of IFN responses in other cell types, we generated stable SLFN2 knockdown NIH3T3 cells via expression of shRNA-targeting SLFN2 using the pSIREN Zsgreen retroviral system. SLFN2 expression was selectively knocked down in NIH3T3 cells (Fig. 6, A and B). Cells in which SLFN2 was knocked down exhibited enhanced proliferation compared with their control counterparts (Fig. 6C). IFNα treatment resulted in dose-dependent growth sup-
pression in both NIH3T3 pSIREN Zsgreen Ctrl siRNA and NIH3T3 pSIREN Zsgreen SLFN2-siRNA cells (Fig. 6C). However, in NIH3T3 cells in which SLFN2 was knocked down, IFNα-induced antiproliferative responses were clearly decreased compared with cells expressing SLFN2 (Fig. 6C), indicating that SLFN2 participates in the generation of the growth inhibitory effects of IFNα. On the other hand, SLFN2 knockdown had no effect on IFNα-dependent formation of STAT-containing DNA-binding complexes (Fig. 6, D and E). Similarly, IFNα-dependent Isg15 gene transcription (Fig. 6F) or generation of IFNα-induced antiviral responses (Fig. 6G) were not affected by SLFN2 knockdown. Thus, targeting SLFN2 appears to be impairing IFNα-dependent cell cycle arrest but not IFN-inducible gene transcription or generation of antiviral responses.

To examine whether SLFN2 plays a role in the control of anchorage-independent growth, we assayed transduced NIH3T3 cells for colony formation in soft agar (41). Colony formation was clearly increased in NIH3T3 pSIREN Zsgreen SLFN2-siRNA cells as compared with NIH3T3 pSIREN Zsgreen Ctrl siRNA cells (Fig. 7, A and B). Notably, the colonies from NIH3T3 pSIREN Zsgreen SLFN2-siRNA cells were consistently larger (Fig. 7A), and the numbers of colonies were increased (Fig. 7B) as compared with NIH3T3 pSIREN Zsgreen
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Ctrl siRNA cells. Taken altogether, these data for the first time implicate SLFN2 in the regulation of anchorage-independent growth.

In subsequent studies, we sought to obtain information on the mechanisms by which SLFN2 regulates anchorage-independent cell growth and blocks cell proliferation. Initially, we examined the effects of SLFN2 knockdown on the expression of various key cell cycle regulators. We compared the levels of expression of Cyclin D1, Cyclin D3, CDK4, CDK6, and the CDK inhibitors p27 KIP1 and p15 INK in serum-starved and cycling NIH3T3 control cells or NIH3T3 cells in which SLFN2 was knocked down. As shown in Fig. 8A, stable SLFN2 knockdown in NIH3T3 cells resulted in higher basal Cyclin D1 levels of expression than control cells, whereas Cyclin D1 levels were also consistently higher in cycling SLFN2 knockdown cells compared with control cells (Fig. 8A). On the other hand, Cyclin D3, as well as CDK4 and CDK6, levels were not significantly altered in cells in which SLFN2 was knocked down across the time points analyzed (data not shown). When the levels of expression of the CDK inhibitors p27 KIP1 and p15 INK were assessed, we noticed that unlike p27 expression, which was not consistently altered (data not shown), p15 INK levels were clearly lower in resting and cycling NIH3T3 pSIREN Zsgreen SLFN2-siRNA transfected cells, compared with NIH3T3 pSIREN Zsgreen Ctrl siRNA transfected cells (Fig. 8B). Thus, although additional mechanisms may be involved, these findings suggest that SLFN2 inhibits cell growth and colony formation in part via suppression of cyclin D1 and up-regulation of the CDK inhibitor p15 INK.

To definitively establish the role of SLFN2 in the generation of IFN responses and anchorage-independent growth in nonhematopoietic cells, we stably knocked down SLFN2 in another murine fibroblast cell line, L929. Initially, we examined the IFN-inducible expression of SLFN2 in L929 cells. The cells were treated with mouse IFNα for different times, and the induction of mRNA and protein expression was analyzed. As expected, both SLFN2 mRNA (Fig. 9A) and protein (Fig. 9B) expression were up-regulated in response to IFNα treatment. We then generated stable SLFN2 knockdown L929 cells via expression of shRNA-targeting SLFN2 using the same pSIREN Zsgreen retroviral system we utilized before to knock down SLFN2 in NIH3T3 cells. Green fluorescent L929 pSIREN Zsgreen cells were selected after retroviral transfection and analyzed for SLFN2 expression. As shown in Fig. 9 (C and D), stable SLFN2 expression was selectively knocked down in L929 pSIREN Zsgreen SLFN2-siRNA cells compared with L929 pSIREN Zsgreen Ctrl siRNA cells. We next analyzed IFNα-dependent Isg15 gene transcription (Fig. 9E) in SLFN2 stable knockdown L929 cells, as well as the effects of stable SLFN2 knockdown on IFNα-induced antiproliferative responses (Fig. 9F). Consistent with the results obtained with NIH3T3 cells, L929 cells with stable SLFN2 knockdown showed enhanced proliferation and were less sensitive to the suppressive effects of IFNα compared with their control counterparts (Fig. 9F), whereas IFNα-dependent Isg15 gene transcription was unaltered (Fig. 9E).

We also determined whether SLFN2 knockdown in L929 cells enhances anchorage-independent growth. L929 pSIREN Zsgreen Ctrl-siRNA and L929 pSIREN Zsgreen SLFN2-siRNA cells were plated in a soft agar assay system. Colony formation was analyzed after 11 days of culture. Representative areas of the soft agar plates for NIH3T3 pSIREN Zsgreen control-siRNA and NIH3T3 pSIREN Zsgreen SLFN2-siRNA cells are shown. B, colonies were counted, and the results were expressed as percentages of control of NIH3T3 pSIREN Zsgreen control-siRNA-derived colonies. The data shown represent the means ± S.E. of three independent experiments, including the one shown in A. Paired t test analysis showed a p value of 0.01.
FIGURE 9. Stable knockdown of SLFN2 enhances cell proliferation and impairs IFN-α-dependent growth inhibitory responses. A, L929 cells were treated with IFNα for 3 or 6 h or left untreated as indicated. Total RNA was subsequently isolated, and the expression of SLFN2 mRNA was analyzed by real time RT-PCR, using specific primers for SLFN2 and GAPDH as an internal control. The data are expressed as fold increases over untreated samples and represent the means ± S.E. of four independent experiments. B, L929 cells were either left untreated or were treated with IFNα for 24 or 48 h, as indicated. After cell lysis, the proteins were resolved by SDS-PAGE and immunoblotted with anti-SLFN2 or anti-GAPDH antibodies, as indicated. C, expression of SLFN2 or SLFN3 mRNAs in L929 Zsgreen-Ctrl siRNA and pSIREN Zsgreen SLFN2-siRNA in L929 cells was determined by real time RT-PCR using specific primers and GAPDH as an internal control. The data are presented as percentages of expression in pSIREN Zsgreen control-siRNA cells and represent means ± S.E. of five experiments. D, total cell lysates from pSIREN Zsgreen SLFN2-siRNA or pSIREN Zsgreen control-siRNA L929 cells were resolved by SDS-PAGE and immunoblotted sequentially with anti-SLFN2 or anti-GAPDH antibodies. E, wild-type L929 cells, pSIREN Zsgreen SLFN2 siRNA L929 cells or pSIREN Zsgreen control-siRNA L929 cells were treated with IFNα for the indicated times. Expression of Isg15 mRNA was determined by real time RT-PCR using GAPDH as an internal control. The data are expressed as fold increases over control untreated cells and represent the means ± S.E. of four experiments. F, equal numbers of L929-pSIREN Zsgreen SLFN2-siRNA or L929-pSIREN Zsgreen control-siRNA cells were either left untreated or treated with the indicated doses of mouse IFNα for 5 days, and cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assays. The means ± S.E. of three experiments are shown.
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The family of Schlafen genes was originally identified during screening for growth regulatory genes that are differentially expressed during lymphocyte development (20). Originally, SLFN family members 1, 2, 3, and 4 were identified and studied (20). Initial studies had suggested that SLFN genes suppress growth and participate in the maintenance of the quiescent state of naive T lymphocytes, as shown by experiments involving ectopic expression of SLFN1, demonstrating disruption of thymic development (20). Subsequently, and based on sequence homology, Geserick et al. (21) identified additional SLFN genes (SLFN5, SLFN8, SLFN9, and SLFN10) forming a cluster on mouse chromosome 11 where the SLFN1–4 genes are also located.

The different members of the SLFN family of proteins can be classified into three subgroups (20, 21). The first group includes SLFN1 and SLFN2, which encode for the smallest two SLFN proteins, with predicted molecular masses of 37 and 42 kDa, respectively (20). They contain an AAA domain, found in ATPases (42), and an adjacent “SLFN box,” which is common to all SLFN proteins (21, 25). Overexpression of SLFN1 results in potent growth suppression by inducing G1 cell cycle arrest (20) through inhibition of cyclin D1 expression (22). In addition, it appears that accumulation of SLFN1 protein to the nucleus correlates with induction of its growth-suppressive effects (43).

The second group of SLFN proteins includes SLFN3 and SLFN4, which have predicted molecular masses of 58 and 68 kDa, respectively. These proteins have in their structures a small sequence motif (SWA(L/V)DL) (21, 25), also shared by the third group. This third group of SLFN proteins contains a Superfamily I DNA/RNA helicase motif not found in group I/II SLFNs, whereas the members of this group are significantly larger proteins with molecular masses ranging between 100 (SLFN5) and 104 kDa (SLFN8) (21). Although the roles of members of this SLFN group remains to be established, studies with SLFN transgenic mice have suggested an important regulatory role for this SLFN gene in T cell development and differentiation (21). Notably, different SLFN family members have been shown to be induced in response to a wide variety of stimuli, including CpG-DNA (24), the bacterial pathogens Brucella and Listeria (44), and terminal differentiation of myeloid cells (21), suggesting that signals from divergent stimuli converge on SLFN family members to control cell cycle progression.

Despite the fact that studies on the functional relevance and biochemical activities of SLFN proteins have been very limited so far, the emerging evidence suggests key regulatory roles for these proteins on cell cycle progression and growth arrest. Yet very little is known about their potential involvement in the generation of the suppressive effects of growth inhibitory cytokines. Type I IFNs are probably the most prominent cytokines that generate growth inhibitory and antitumor effects; and these properties have over the years led to their introduction in the treatment of various leukemias and solid tumors (3). Importantly, although it is well established that IFNs regulate cell cycle progression and induce G0/G1 cell cycle arrest, very little is known about the IFN-inducible proteins that mediate such responses. In the present study, we provide the first evidence that IFNs regulate expression of members of the SLFN family of genes and proteins. Our data demonstrate that IFNα is a potent inducer of different SLFN family members, including members of Group I (SLFN1 and SLFN2), Group II (SLFN3), and Group III (SLFN5 and SLFN8). Moreover, in work aimed to define the regulation of expression of these proteins by IFNs, we established the differential involvement of distinct IFN-activated STAT proteins and the p38 MAP kinase in their regulation.

Our finding that members of the SLFN family of proteins are engaged by the Type I IFN receptor in a STAT- and/or p38

FIGURE 10. Effects of SLFN2 knockdown on anchorage-independent growth of L929 cells. A, equal numbers of L929 pSIREN Zsgreen control-siRNA and L929 pSIREN Zsgreen SLFN2-siRNA cells were plated in a soft agar assay system. Colony formation was analyzed after 8 days of culture. Representative areas of L929 pSIREN Zsgreen SLFN2-siRNA cells were plated, and colony formation was determined after 8 days of culture in soft agar. As depicted in Fig. 10, L929 cells with stable SLFN2 knockdown showed consistently larger colonies (Fig. 10A), and there were increased numbers of colonies compared with L929 pSIREN Zsgreen Ctrl siRNA cells (Fig. 10B).

Discussion

The family of Schlafen genes was originally identified during screening for growth regulatory genes that are differentially expressed during lymphocyte development (20). Originally, SLFN family members 1, 2, 3, and 4 were identified and studied (20). Initial studies had suggested that SLFN genes suppress growth and participate in the maintenance of the quiescent state of naive T lymphocytes, as shown by experiments involving ectopic expression of SLFN1, demonstrating disruption of thymic development (20). Subsequently, and based on sequence homology, Geserick et al. (21) identified additional SLFN genes (SLFN5, SLFN8, SLFN9, and SLFN10) forming a cluster on mouse chromosome 11 where the SLFN1–4 genes are also located.

The different members of the SLFN family of proteins can be classified into three subgroups (20, 21). The first group includes SLFN1 and SLFN2, which encode for the smallest two SLFN proteins, with predicted molecular masses of 37 and 42 kDa, respectively (20). They contain an AAA domain, found in ATPases (42), and an adjacent “SLFN box,” which is common to all SLFN proteins (21, 25). Overexpression of SLFN1 results in potent growth suppression by inducing G1 cell cycle arrest (20) through inhibition of cyclin D1 expression (22). In addition, it appears that accumulation of SLFN1 protein to the nucleus correlates with induction of its growth-suppressive effects (43).

The second group of SLFN proteins includes SLFN3 and SLFN4, which have predicted molecular masses of 58 and 68 kDa, respectively. These proteins have in their structures a small sequence motif (SWA(L/V)DL) (21, 25), also shared by the third group. This third group of SLFN proteins contains a Superfamily I DNA/RNA helicase motif not found in group I/II SLFNs, whereas the members of this group are significantly larger proteins with molecular masses ranging between 100 (SLFN5) and 104 kDa (SLFN8) (21). Although the roles of members of this SLFN group remains to be established, studies with SLFN transgenic mice have suggested an important regulatory role for this SLFN gene in T cell development and differentiation (21). Notably, different SLFN family members have been shown to be induced in response to a wide variety of stimuli, including CpG-DNA (24), the bacterial pathogens Brucella and Listeria (44), and terminal differentiation of myeloid cells (21), suggesting that signals from divergent stimuli converge on SLFN family members to control cell cycle progression.

Despite the fact that studies on the functional relevance and biochemical activities of SLFN proteins have been very limited so far, the emerging evidence suggests key regulatory roles for these proteins on cell cycle progression and growth arrest. Yet very little is known about their potential involvement in the generation of the suppressive effects of growth inhibitory cytokines. Type I IFNs are probably the most prominent cytokines that generate growth inhibitory and antitumor effects; and these properties have over the years led to their introduction in the treatment of various leukemias and solid tumors (3). Importantly, although it is well established that IFNs regulate cell cycle progression and induce G0/G1 cell cycle arrest, very little is known about the IFN-inducible proteins that mediate such responses. In the present study, we provide the first evidence that IFNs regulate expression of members of the SLFN family of genes and proteins. Our data demonstrate that IFNα is a potent inducer of different SLFN family members, including members of Group I (SLFN1 and SLFN2), Group II (SLFN3), and Group III (SLFN5 and SLFN8). Moreover, in work aimed to define the regulation of expression of these proteins by IFNs, we established the differential involvement of distinct IFN-activated STAT proteins and the p38 MAP kinase in their regulation.

Our finding that members of the SLFN family of proteins are engaged by the Type I IFN receptor in a STAT- and/or p38

FIGURE 10. Effects of SLFN2 knockdown on anchorage-independent growth of L929 cells. A, equal numbers of L929 pSIREN Zsgreen control-siRNA and L929 pSIREN Zsgreen SLFN2-siRNA cells were plated in a soft agar assay system. Colony formation was analyzed after 8 days of culture. Representative areas of L929 pSIREN Zsgreen SLFN2-siRNA cells were plated, and colony formation was determined after 8 days of culture in soft agar. As depicted in Fig. 10, L929 cells with stable SLFN2 knockdown showed consistently larger colonies (Fig. 10A), and there were increased numbers of colonies compared with L929 pSIREN Zsgreen Ctrl siRNA cells (Fig. 10B).
MAPK-dependent manner provided a direct link between IFN-activated Jak-STAT pathways and cellular elements controlling cell cycle progression. Such a link led us to further studies aimed to define the functional relevance of the SLFN pathway in the generation of IFN responses. We focused our efforts on SLFN2, a member of group I SLFNs, whose expression was greatly induced by activation of the Type I IFN receptor. Selective knockdown of this protein resulted in enhanced bone marrow-derived hematopoietic progenitor cell growth, whereas IFN-dependent suppression of normal hematopoietic progenitor colony formation was less noticeable in such cells. Nevertheless, it is possible that some functional redundancy among different SLFN members may account for residual IFN-dependent hematopoietic suppression in the absence of SLFN2. Stable knockdown of SLFN2 also substantially diminished the ability of IFNα to generate antiproliferative responses in non-hematopoietic cells, strongly implicating this protein in the generation of the growth-suppressive effects of IFNs. Our data also suggest for the first time an important role for SLFN2 in the control of anchorage-independent cell growth, whereas we did not find any requirement for this protein in the generation of IFN-dependent antiviral effects. There was also no requirement for SLFN2 in Type I IFN-dependent formation of STAT-binding complexes or IFN-inducible transcription of Isg15. Thus, it appears that this member of the SLFN family of proteins specifically mediates signals that participate in the induction of the growth-suppressive effects of IFNs but not their antiviral effects.

Our studies establish that beyond engagement of SLFN2, IFNα up-regulates expression of several other members of the SLFN family. The functional differences among distinct SLFN groups and individual SLFN members in the generation of IFN responses remain to be established. Nevertheless, the Type I IFN-dependent induction of expression of several members of this family raises the possibility that beyond SLFN2, other members participate in the generation of IFN-inducible responses, but this remains to be directly determined in future studies. Interestingly, the SLFN gene cluster was recently linked to development of rheumatoid arthritis by combining microarray analyses of two independent rheumatoid arthritis mouse models (45). Because overproduction of various cytokines is linked to the pathogenesis of rheumatoid arthritis (31), these data raise the possibility that beyond IFNs, SLFN genes and their products may be involved in cellular pathways activated by several other cytokines, and this needs to be examined in future work.

The involvement of SLFN2, and possibly other Schlafens, in the control of IFNα-antiproliferative effects may ultimately prove to be of clinical-translational therapeutic relevance. IFNα exhibits potent antineoplastic properties in vitro and in vivo, and it has substantial clinical activity in the treatment of various malignancies. However, a limiting factor in the administration of higher, more effective, doses of IFNs has been the various side effects that reflect the diversity of responses elicited by these pleiotropic cytokines. It is possible that SLFN genes and their products selectively mediate the antiproliferative effects of IFNs, because different groups of genes are mediators of IFN-regulated antiviral effects (32). Moreover, it is conceivable that development of methodologies to selectively induce SLFN gene expression may specifically promote the antitumor effects of IFNs in the absence of engagement of other pathways associated with various IFN-inducible adverse effects. Although the validity of such a hypothesis remains to be determined, further work in this direction is warranted and may provide interesting new information and help in attempts to optimize the antitumor effects of IFNα.

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